Journal of Scientific & Industrial Research
Vol. 75, October 2016, pp. 609-614

Bioprocess Development for β- and γ-rubromycin Production: A Human Telomerase Inhibitors, by *Streptomyces* sp. ADR1

A Z Boumehira[1,2], R A Malek[3], N Z Othman[3], I Ware[3], S Ramli[3], K Malek[3], H Hacène[2] and H A El-Enshasy[3,4*]

[1]Centre de Recherche Scientifique et Technique en Analyses Physico Chimiques, Bou-Ismail, Tipaza, Algeria
[2]University of Sciences and Technology Houari Boumediene, FSB, LBCM, Bab Ezzouar, Algiers, Algeria
[3]Institute of Bioproduct Development, Universiti Teknologi Malaysia (UTM), Johor 81310, Malaysia
[4]City of Scientific Research and Technology Applications, New Burg Al Arab, Alexandria 21934, Egypt

Received 03 June 2015; revised 25 May 2016; accepted 26 July 2016

Rubromycins are important group of antibiotic produced by actinomycetes. For many years this telomerase inhibitor anticancer bioactive compound was mainly produced by either microbes belongs to actinomycetes or by mean of chemical synthesis. In this work, we used a new thermotolerant rubromycins producer strain (*Streptomyces* sp. ADR1) isolated from Algerian desert. This strain was able to produce a mixture of rubromycins (β and γ), at relatively high concentrations. At first, the most suitable medium composition for production process was selected through screening of different medium used for secondary metabolites production by actinomycetes followed by complete studies on growth and production kinetics in shake flask level. At this stage, the maximal rubromycins production were 24.58 mg/L and 356 mg/L, for β-rubromycins and γ-rubromycins, respectively. After this step, cultivations were conducted in 16-L stirred tank bioreactor for further study the industrial potential of this process. The results showed that transferring the process from shake flask to bioreactor level and cultivation under non-oxygen limitation increased the volumetric production up to 27.41 mg/L, and 580.35 mg/L for β-rubromycins and γ-rubromycins, respectively.

Keywords: *Streptomyces* sp. ADR1, Human Telomerase Inhibitor, β-rubromycin, Anticancer Drugs, Algerian Sahara Desert.

Introduction

Cancer treatment represents a real challenge for the international community. 8.2 million cancer deaths were registered in 2012. Therefore, cancer is one of the major reason causing death in the world, and the development of new anticancer drugs is one of the interesting point of research[1-4]. Telomerase based anticancer drugs have recently attract a special attention. This enzyme is expressed in 80–85 % of the tumor cells, and is not expressed in the most normal human cells[5-7], which promotes its position as a selective target in the treatment of cancer associated with the high expression of telomerase. Another advantage, is that telomerase based cancer therapies develop less resistance to chemotherapy compared to drugs based on growth factor receptors or signal transduction enzymes target in cancer cells[7,8]. Quinone antibiotics β- and γ- rubromycin are a good human telomerase inhibitor with IC_{50} values from 2.64–12.2 μM[9,10]. This activity is due to spiroketal moiety of rubromycins which plays an important role in the telomerase inhibition process[7,9,11,12]. Actinomycetes are considered as one of the main biofactory for the production of many important antibiotics of different groups[13-15]. Therefore, isolation and identification of new antibiotics or production of known antibiotic in high concentration using new isolates are still important research topic. In this paper, we describe the fermentation process of the production of the human telomerase inhibitors β- and γ-rubromycin, using *Streptomyces* sp. ADR1, a new strain isolated from the Algerian sahara desert.

Experimental section

Microorganism

The β- and γ-rubromycin producing strain, *Streptomyces* sp. ADR1, was isolated from soil collected in Algeria the Algerian Sahara Desert (26.698 N 0.1086 E). The strain was identified to belong to the genus *Streptomyces*. The partial 16S ribosomal RNA gene sequence of the strain *Streptomyces* sp. ADR1 (1392 bp) is deposited in the GenBank under Accession No. KF947515 and deposited in the Wellness Industries Culture

*Author for correspondence
E-mail: henshasy@ibd.utm.my

Collection (WICC), of Institute of Bioproduct Development, UTM, Malaysia, under the number (WICC- B86).

Inoculum preparation

From the master cell bank culture (preserved in 50% glycerol ($v.v^{-1}$) at -80 °C), strain was first activated on ISP2 agar[16] and incubated for 10 days for full sporulation on agar plate. The obtained spores were collected in glycerol/distilled water solution (50%), and stored at -80 °C as working cell bank.

Each experiment was started with inoculation of 250 mL Erlenmeyer flasks (working volume of 50 mL) with 2 ml of spore suspension (working cell bank). The inoculated flasks were then incubated at 200 rpm and 28°C for 48 h on rotary shaker (Innova 4080, New Brunswick Scientific, NJ, USA). The antibiotic production media, either in Erlenmeyer flasks or in stirred tank bioreactor, were then inoculated with 10% ($v.v^{-1}$) of the 48 h vegetative broth.

Media screening for cell growth and rubromycins production

Streptomyces sp. ADR1 was cultivated on eight different media for the evaluation of the best medium for cell growth and rubromycin production. The composition of media was as follows in (g/L): Medium 1: glucose, 4.0; yeast extract, 4.0 and malt extract, 10.0[16], Medium 2: dextrin, 70.0; soybean flour, 35.0; corn steep liquor, 5.0; DL-methionine, 2.0; $CaCO_3$, 5.0, $CoCl_2$, 0.004 and $MnSO_4.7H_2O$, 0.1 [17], Medium 3: sucrose, 50.0; yeast extract, 10.0; $NaNO_3$, 30.0; K_2HPO_4, 10.0; $MgSO_4$, 0.5; $FeSO_4$, 0.01 and KCl, 0.5[18], Medium 4: glucose, 15.0; yeast extract, 1.0; soybean meal, 15.0; NaCl, 5.0; $CaCO_3$, 1.0 and Glycerol, 2.5mL[19], Medium 5: starch, 7.5; soybean meal, 4.0; $CaCO_3$, 4.0; K_2HPO_4, 0.9; $FeSO_4$, 0.03 and $CoCl_2$, 0.001[20], Medium 6: starch, 10.0; $(NH_4)_2SO_4$, 2.0; $CaCO_3$, 2.0; NaCl, 1.0; K_2HPO_4, 1.0; $MgSO_4$. $7H_2O$, 1.0; $FeSO_4 \cdot 7H_2O$, 0.001; $MnCl_2 \cdot 4H_2O$, 0.001 and $ZnSO_4 \cdot 7H_2O$, 0.001[16], Medium 7: Starch, 10.0; Pharmamedia (Amino. Acids), 5; $CaCO_3$, 1.0; NaI, 0.0005 and $CuSO_4$. $5H_2O$, 0.05[21], Medium 8: starch, 10.0; glycerol, 10.0 and $(NH_4)_2SO_4$, 3.0[22]. The pH of all media was adjusted to 7.0-7.2. The inoculated flasks were incubated on the rotary shaker (Innova 4080, New Brunswick, NJ, USA) at 200 rpm and 28 °C for 14 days. At the end of the fermentation, Cell Dry Weight (CDW), β- and γ-rubromycin production, and final pH were determined for each medium.

Kinetics of production of β- and γ-rubromycin in shake flasks

In shake flask experiment, 250 flasks with 50 mL working volume were inoculated and incubated on the rotary shaker (Innova 4080, New Brunswick, NJ, USA) at 200 rpm and 28°C for 15 days. Samples were collected every day in falcon tube and used for cell dry weight determination, rubromycins production and glucose determination.

Production of β- and γ-rubromycin in 16-L stirred tank bioreactor

The fermentation medium chosen was medium 1, pH: 7.2, and fermentation process was carried out using 16-L stirred tank bioreactor (BioEngineering AG, Wald, Switzerland) with working volume of 10 L. The stirrer was equipped with two 6-blade Rushton turbine impellers (d_i(impeller diameter)= 85 mm; d_t(tank diameter)= 214 mm, d_i/d_t= 0.397). The bioprocess was in uncontrolled pH mode, with an initial pH= 7.00. Dissolved oxygen was adjusted to 100% saturation before inoculation and kept uncontrolled during cultivation process. The pH and Dissolved Oxygen concentration (DO) were determined using pH and DO polarographic electrodes (Ingold, Mittler-Toledo, Switzerland). During the experiments, temperature, aeration rate and the agitation speed were controlled at 28ºC, 1 vv^{-1} min^{-1} and 400 rpm, respectively. The total time of fermentation was 5 days. Samples of 50mL each, were collected every 6 hours in falcon tube and used for cell dry weight, rubromycins, and total reducing sugar determinations.

Growth measurement, Cell Dry Weight (CDW) Determination

For the Cell Dry Weight (CDW) determination, samples were centrifuged at 5000 rpm for 20 min. After that the cells were washed twice using distilled water, followed by centrifugation. The centrifuge tubes were then dried to a constant weight at 80 °C for 24 hours to determine the dry cell weight. A small fraction of the supernatant was frozen at −20 °C for reducing sugar and rubromycins determination.

Analysis

Determination of β- and γ-rubromycin production

β- and γ-rubromycin were analysed using a Waters HPLC system (Milford, MA, USA) consisting of a pump and system controller (Model Waters e2695) and photo-diode array detector (Model 2998). These compounds separation was done by a C18 reserved phase Xbridge column (5 μm, 54.6 × 250 mm) at 25°C with a flow rate of 0.8 ml/min. The mobile phase was consisted of 0.1% of acetic acid (40%) / Acetonitrile (60%), in an isocratic programme. The injection volume is 20 μL. All samples were filtered

with 0.45 µm nylon filters prior to injection. The detection was monitored at 490 nm and data were integrated by Empower 3 software (Waters) (Milford, MA, USA)

Determination of reducing sugar in fermentation broth

Residual reducing sugar concentration in fermentation broths was determined as method described by Miller [23]. This method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. The chemical method is based on the determination of the color developed after the reaction between the reduced sugar and 3, 5- Dinitrosalisylic acid (DNS) in citrate buffer solution. DNS analysis was performed by adding 50 µL of sample to 600µL of DNS solution (prepared by dissolving 10g 3, 5-Dinitrosalisylic acid, 2g phenol, 0.9 sodium sulphate and 10g sodium hydroxide in 1 L water) and 350 µL of citrate buffer in a clean test tube. After vortex, the samples were incubated in a 95 °C water bath for 5 min. Later, samples were instantly placed into an ice bath for 03 min. The absorbance was determinated at 540 nm using SPECTRONIC™ 200 (Thermo Fisher Scientific, USA) Spectrophotometer. Standard glucose solutions with appropriate amounts are added to the test to draw the calibration curve of absorbance vs. glucose concentration. The glucose concentration was expressed in g/L.

Results and discussion

Media screening for rubromycins production

In this experiment, different media from literatures were applied to investigate their ability to support cell growth and rubromycins production by the newly isolated strain *Streptomyces* sp. ADR1. Therefore, eight different media which were reported before to support antibiotic production by actinomycetes were applied in this experiment. Cultivations were conducted in parallel in shake flask level in triplicate. As shown in figure 1, different media supported cell growth and rubromycins production in different extent. It was clearly observed that the maximal antibiotic production of 22.7 mg/L and 350.13 mg/L for β-rubromycin and γ-rubromycin, respectively, was obtained in medium No. 1 which composed of only malt extract, yeast extract, and glucose. This was followed by medium No. 4, No. 6, No. 5, and No. 7. Other medium were very weak on supporting the antibiotic production. On the other hand, the maximal biomass production of 3.58 g/L was obtained in medium 4 followed by Medium 1 and 7. For better understanding the cell performance for rubromycins production in different media, yield coefficient in terms of specific rubromycins production was calculated. The maximal values for both $Y_{\beta/X}$ and $Y_{\gamma/X}$ which present the amount of rubromycins β and γ produced per unit biomass were 105.74 [mg/g] and 6.86 [mg/g], respectively. This indicates that this medium not only supported volumetric production but also the higher cell performance for antibiotic production. Therefore, the growth and antibiotic production kinetic studies were further conducted in shake flask and bioreactor cultures using this medium. The suitability of this medium for antibiotic production is due to its balanced nutrient composition with rich components which support secondary metabolites production. In other studies, this medium was also supported other antibiotics produced by actinomycetes such as oxytetracyclines and many other antibiotics. The existence of yeast extract is also necessary to support secondary metabolites

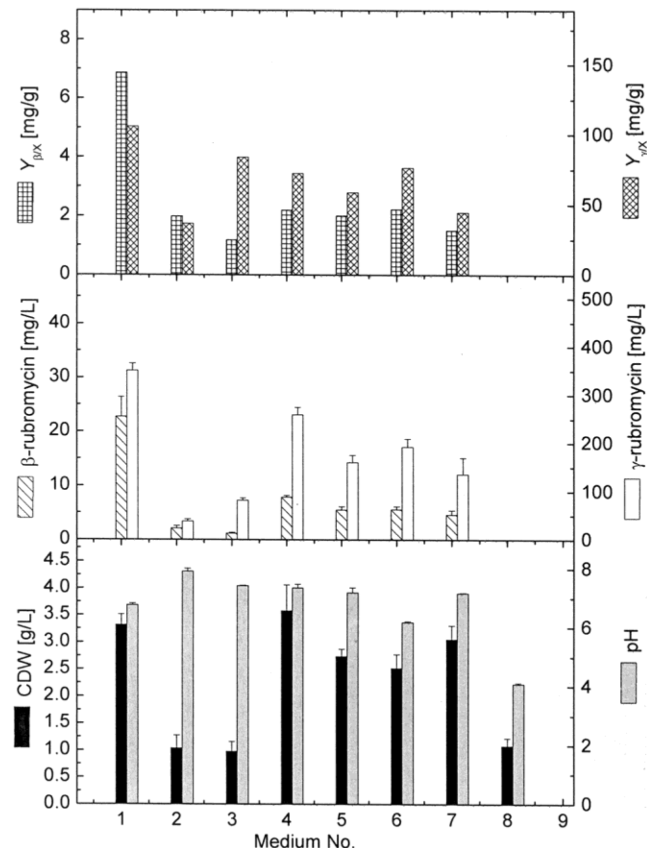

Fig. 1—Effect of different media on cell growth, volumetric and specific rubromycins production when *Streptomyces* sp. ADR1 cultivated in different media in shake flask level. Data were taken after 14 days cultivation and the error bar indicate standard deviration from three different flasks.

production. Yeast extract is not only considered as source of nitrogen but also for vitamins and may growth factors which act as limiting factors for different antibiotic biosynthetic pathways[24-26].

Kinetics of cell growth and rubromycins production in shake flask

The cell growth and rubromycins production kinetics in shake flask level during cultivation for 360 hours are presented in figure 2. As shown in this figure, cells grew exponentially without significant lag phase with rate of 0.013 g/L/h, reaching the biomass of 3.2 g/L after 240 h of cultivation. Once cells entered the stationary phase, the biomass kept more or less constant for the rest of cultivation time. During the active growth phase, the reducing sugar consumption was reduced from its initial value of 5.1 g/L to about 0.5 g/L with consumption rate of 0.017 g/L/h. It is also worthy to note that pH in culture was slightly dropped from 7 to 6.5 during the active growth phase and increased gradually thereafter as cell entered the stationary phase, reaching almost 7 at the end of cultivation time. As also shown in the same figure, both of β and γ rubromycins were not detected in culture during the first 144 hours. After that time, β-rubromycin was first detected in culture broth and increased gradually reaching its maximal titer of 24.58 mg/L after 336 h.

Kinetics of cell growth and rubromycins production in bioreactor level

In this experiment, cells were cultivated in 16-L stirred tank bioreactor using the same medium composition and inoculum size used in the shake flask experiments. Cultivations were performed in 16-L stirred tank bioreactor of 10-L working volume under uncontrolled pH condition. The concentration profile of cell dry weight, total carbohydrates, and rubromycins production are presented in figure 3. As

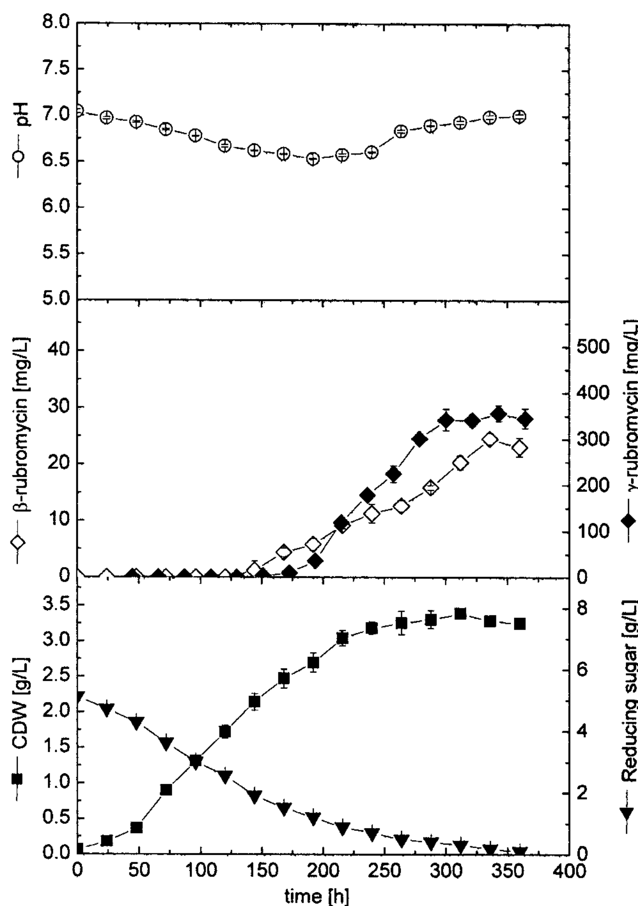

Fig. 2—Kinetics of cell growth, sugar consumption, rubromycins production, and pH changes during cultivation of *Streptomyces* sp. ADR1 in shake flasks level. (Data presented are the average of three different flasks.

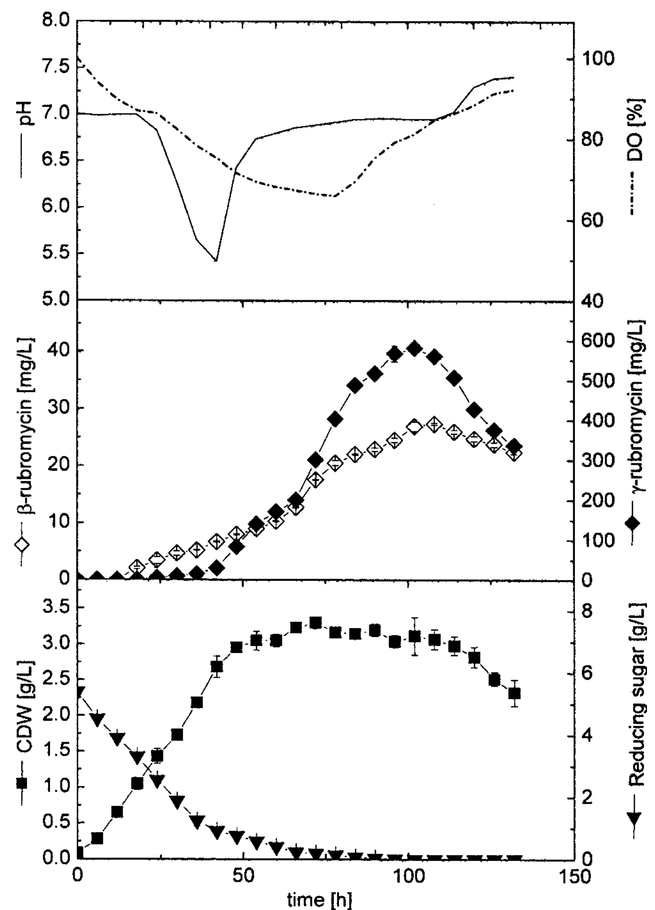

Fig. 3—Kinetics of cell growth, sugar consumption, rubromycins production, pH and DO change during cultivation of *Streptomyces* sp. ADR1 in 16-stirred tank bioreactor. (Data presented are the average of two parallel bioreactor cultivations).

shown, cells grew exponentially without lag phase with rate of 0.056 g/L/h. This value was almost 3.7 times compared to the growth rate in shake flask. A maximal cell dry weight of 3.30 g/L was obtained in bioreactor culture after only 72 hours. Thus, cultivation in bioreactor reduced the growth phase by 240 h compared to shake flask. The higher growth rate in bioreactor culture is attributed to the better mixing and oxygen transfer rate in bioreactor. This significant reduction in growth phase in case of cultivation of aerobic microorganisms in bioreactor was also reported in other cultivation processes as well[27]. During this active growth phase, the sugar concentration was reduced gradually in culture with rate of 0.083 g/L/h and reached almost 0.2 g/L as cells entered the stationary phase. The biomass was kept more or less constant between 72 and 108 hours, after that time cell concentration decreased gradually in culture with rate of 0.031 g/L/h and reached 2.32 g/L after 132 h. However, this decrease in biomass in shake flask was not observed. The decrease in cells in bioreactor culture may attributed to the higher shear stress compared to shake flask as also reported in cultivation of other actinomycetes such as *S. natalensis*[14] and *Saccharopolyspora erhythraea*[13] and other filamentous microorganisms. However, during the growth phase, DO decreased in parallel to cell growth and reached its minimal value of 65.9% saturation and increased again thereafter. The value of DO was almost 80% saturation during the stationary phase and decreased again gradually once cells entered the decline phase. Production of rubromycins was not detected in culture during the first 18 h of cultivation. However, β-rubromycin production started after 18 h and increased gradually in culture with rate of 0.31 mg/L/h, reaching its maximal value of 27.41 mg/L after 108 h cultivation. This value was close to those obtained in shake flask culture. On the other hand, the production of γ-rubromycin was first detected 6 hours later (after 24 h cultivation) and increased gradually in culture with rate of 8.58 mg/L/h, reaching maximal value of 580.35 mg/L after 102 h. It was observed that, this antibiotic was not stable in culture and its concentration decreased gradually with rates of 0.21 mg/L/h and 8.13 mg/L/h, for β- rubromycin and γ-rubromycin, respectively. The degradation of antibiotic after reaching its maximal production was also reported for other antibiotics such in case of rifamycins, natamycins, and cyclosporine[13,25,28]. This was attributed to carbon source limitation and thus the antibiotic could be re-consumed as source for maintenance energy or as a result of degradation by specific enzymes produced in culture during the decline growth phase of cells.

Table 1—Kinetics of cell growth and rubromycins production during batch cultivation of *Streptomyces* sp. ADR1 in shake flasks and in stirred tank bioreactors.

Parameters	Shake flask	Bioreactor
Growth Parameters		
X_{max} [g/L]	3.39 (312 h)	3.30 (72 h)
dx/dt [g/L/h]	0.015	0.056
μ [h^{-1}]	0.016	0.037
Production Parameters		
Rubromycin β_{max} [mg/L]	24.58 (336 h)	27.41 (108 h)
Rubromycin γ_{max} [mg/L]	356.00 (336 h)	580.35 (102 h)
$Q_{Rub\beta}$ [mg/L/h]	0.13	0.31
$Q_{Rub\gamma}$ [mg/L/h]	1.91	8.58
Q_s [g/L/h]	0.016	0.083
$-Q_{Rub\beta}$ [mg/L/h]	nil	0.21
$-Q_{Rub\gamma}$ [mg/L/h]	nil	8.13
$Y_{\beta/X}$ [mg/g]	7.25	7.40
$Y_{\gamma/X}$ [mg/g]	105.01	176.12

X_{max}: maximal cell dry weight, dx/dt: growth rate; μ: specific growth rate, Rubromycin β_{max}: maximal β-rubromycin production, Rubromycin γ_{max}: maximal γ-rubromycin production, $Q_{Rub\beta}$: β-rubromycin production rate, $Q_{Rub\gamma}$: γ-rubromycin production rate, Q_s: reducing sugar consumption rate, $-Q_{Rub\beta}$: β-rubromycin degradation rate, $Q_{Rub\gamma}$: γ-rubromycin degradation rate, $Y_{\beta/X}$: yield of β-rubromycin on biomass, $Y_{\gamma/X}$: yield of γ-rubromycin on biomass.

Conclusion

Table 1 summarizes the kinetics of cell growth, sugar consumption, and rubromycins production when cultivations carried out in shake flask and in bioreactor cultures. It is clearly observed that cultivation in bioreactor yielded almost the same biomass but with significant increase in rubromycins production. It is also interesting to report that, antibiotic production time was significantly reduced from 336 h in shake flask to only 108 h in bioreactor. This directly related to the continuous aeration and better mixing in the bioreactor. The process developed in this study shows high potential future application for industrial production of this important antibiotic.

Acknowledgements

This work was supported by the project No. 284/SA/2012, Centre de Recherche Scientifique et Technique en Analyses Physico-Chimiques – C.R.A.P.C. Algeria, and the Algerian scholarship

program « Programme de formation résidentielle à l'étranger au titre de l'année universitaire 2014-2015 », and the Institute of Bioproduct Development, Universiti Teknologi Malaysia (UTM), Johor, Malaysia.

References

1 International Agency for Research on Cancer, *World cancer report 2014*, edited by B Stewart & Wild C P, IARC, Lyon (2015).

2 Tabernero J & Board E E, Proven efficacy, equitable access, and adjusted pricing of anti-cancer therapies: no 'sweetheart' solution, *Ann Oncol*, **26** (2015) 1529-1531.

3 Siegel R L, Miller K D & Jemal A, Cancer statistics, 2016, *CA Cancer J Clin,* **66** (2016) 7-30.

4 Chen W, Zheng R, Baade P D, Zhang S, Zeng H, Bray F, Jemal A, Yu X Q & He J, Cancer statistics in China, 2015, *CA Cancer J Clin,* **66** (2016) 115–132.

5 Shay J W & Wright W E, Role of telomeres and telomerase in cancer, *Semin Cancer Biol,* **21** (2011) 349-353.

6 Flores I, Benetti R & Blasco M A, Telomerase regulation and stem cell behaviour, *Curr Opin Cell Biol,* **18** (2006) 254-260.

7 Kiran K G, Palaniswamy M & Angayarkanni J, Human telomerase inhibitors from microbial source, *World J Microbiol Biotechnol,* **31** (2015) 1329-1341.

8 Harley C B, Telomerase and cancer therapeutics, *Nat Rev Cancer*, **8** (2008) 167-179.

9 Ueno T, Takahashi H, Oda M, Mizunuma M, Yokoyama A, Goto Y, Mizushina Y, Sakaguchi K & Hayashi H, Inhibition of human telomerase by rubromycins: Implication of spiroketal system of the compounds as an active moiety, *Biochemistry,* **39** (2000) 5995-6002.

10 Mizushina Y, Takeuchi T, Sugawara F & Yoshida H, Anti-cancer targeting telomerase inhibitors: beta-rubromycin and oleic acid, *Mini-Rev Med Chem,* **12** (2012) 1135-1143.

11 Brasholz M, Sorgel S, Azap C & Reissig H U, Rubromycins: Structurally intriguing, biologically valuable, synthetically challenging antitumour antibiotics, *Eur J Org Chem*, (2007) 3801-3814.

12 Willis N J & Bray C D, An ortho-quinone methide based strategy towards the rubromycin spiroketal family, *Rsc Adv,* **5** (2015) 80212-80215.

13 El-Enshasy H A, Mohamed N A, Farid M A & El-Diwany A I, Improvement of erythromycin production by *Saccharopolyspora erythraea* in molasses based medium through cultivation medium optimization, *Bioresour Technol*, **99** (2008) 4263-4268.

14 Elsayed E A, Farid M A F & El Enshasy, H A, Improvement in natamycin production by *Streptomyces natalensis* with the addition of short-chain carboxylic acids, *Process Biochem*, **48** (2013) 1831-1838.

15 Boumehira A Z, El-Enshasy H A, Hacène H, Elsayed E A, Aziz R & Park E, Y, Recent progress on the development of antibiotics from the genus *Micromonospora*, *Biotechnol Bioprocess Eng,* **21** (2016) 199-223.

16 Shirling E B & Gottlieb D, Methods for characterization of *Streptomyces* species, *Int J Syst Evol Microbiol,* **16** (1966) 313-340.

17 Gil G H, Park S B, Lim J Y, Lee J H & Yoo M Y, Effects of aeration and agitation on the sisomicin production by *Micromonospora inyonensis*, *Korean J of Chem Eng,* **5** (1988) 19-22.

18 Chen C, Song F, Wang Q, Abdel-Mageed W M, Guo H, Fu C, Hou W, Dai H, Liu X & Yang N, A marine-derived *Streptomyces* sp. MS449 produces high yield of actinomycin X2 and actinomycin D with potent anti-tuberculosis activity, *Appl Microbiol Biotechnol*, **95** (2012) 919-927.

19 Atlas R M, *Handbook of Media for Environmental Microbiology*, **4th edn**, CRC Press, Florida (2005), 1645.

20 Meenavilli H, Potumarthi R & Jetty A. Gentamicin production by *Micromonospora echinospora* Me-22 in stirred tank reactor: effect of various parameters, *J Basic Microbiol,* **48** (2008) 53-58.

21 Lam K S, Titus J A, Dabrah T T, Kimball D L, Veitch J M, Gustavson D R, Compton B J, Matson J A, Forenza S & Ross J, Improved processes for the production and isolation of dynemicin A and large-scale fermentation in a 10000-liter fermentor, J Ind Microbiol, **11** (1992) 7-12.

22 Kim B S, Moon S S & Hwang B K, Isolation, antifungal activity, and structure elucidation of the glutarimide antibiotic, streptimidone, produced by *Micromonospora coerulea*, *J Agric Food Chem*, **47** (1999) 3372-3380.

23 Miller G.L, use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal Chem*, **31** (1959) 426-428.

24 Farid M, El-Diwany A, El-Enshasy H A & Abu-Shady M, Production of rifamycin B and SV by free and immobilized cells of *Amycolatopsis mediterranei*, *Acta Biotechnol*, **15** (1995) 241-248.

25 El-Enshasy H A, Beshay U I, El-Diwany A I, Omar H M, El-Kholy A G E & El-Najar R, Improvement of rifamycins production by *Amycolatopsis mediterranei* in batch and fed-batch cultures, *Acta Microbiol Pol*, **52** (2003) 301-313.

26 Abdel-Fattah Y R, El Enshasy A H, Anwar M, Omar H, Abolmagd E & Zahra R A, Application of factorial experimental designs for optimization of cyclosporin A production by *Tolypocladium inflatum* in submerged culture, *J Microbiol Biotechnol,* **17** (2007) 1930-1936.

27 Maftoun P, Malek R, Abdel-Sadek M, Aziz R & El-Enshasy H A, Bioprocess for semi-industrial production of immunomodulator polysaccharide Pleuran by *Pleurotus ostreatus* in submerged culture, *J Sci and Industr Res,* **72** (2013) 655-662.

28 Farid M A, El-Enshasy H A, El-Diwany A I & El-Sayed E A, Optimization of the cultivation medium for natamycin production by *Streptomyces natalensis*, *J Basic Microbiol*, **40** (2000) 157-166.