SENRA Academic Publishers, Burnaby, British Columbia
Vol. 4, No. 1, pp. 1021-1026, 2010
ISSN: 1715-9997

Canadian Journal of pure&applied sciences

LARVICIDAL ACTIVITY OF EXTRACELLULAR SECONDARY METABOLITES OF *STREPTOMYCES MICROFLAVUS* AGAINST *CULEX PIPIENS*

Magda A El-Bendary, *Hala M Rifaat and Abeer A Keera
Department of Microbial Chemistry, National Research Centre, Cairo, Egypt

ABSTRACT

Eight isolates of actinomycetes were isolated from sand samples of underground spring at Giza Governorate, Egypt. The extracellular secondary metabolites of one isolate (Act-1) showed larvicidal activity against *Culex pipiens*. The morphological, physiological and chemotaxonomical characteristics of this isolate revealed that it belongs to *Streptomyces* sp. However, according to the 16S rDNA sequencing analysis, it was identified as *Streptomyces microflavus*. Twenty five ml of peptone-beef extract-yeast extract-glucose medium under static condition showed the best conditions for the production of secondary metabolites from Act-1 against mosquito larva. Addition of NaCl, $FeSO_4$ and $CaCO_3$ to the medium increased the produced mosquitocidal metabolites twelve times more than the original. The LC_{50} value of *Streptomyces microflavus* metabolites was 77.3 ppm with 95% fiducial limits (62.6-95.7).

Keywords: *Culex pipiens*, larvicidal, secondary metabolites, *Streptomyces.*

INTRODUCTION

Mosquitoes are the oldest human enemy and are the most medically important arthropod vectors of disease. Mosquitoes transmit many dreadful diseases like malaria, filariasis, Japanese encephalitis and dengue fever affecting the socio-economical status of many nations (Service, 1983). Mosquitoes are also an important pest of humans, causing allergic responses that include local skin reaction and systemic reaction such as angioedema and urticaria (Peng *et al*., 1999). *Culex pipiens* is the most widely distributed species in the world and is predominantly found in all Egyptian governorates (El-Kady *et al*., 2008).

Actinomycetes and its derived products are highly toxic to mosquitoes, yet have low toxicity to non target organisms (Vijayan and Balaraman, 1991). Accordingly, the use of actinomycetes may be a promising approach for biological control of mosquitoes (Liu *et al*., 2008). The filamentous actinomycetes are Gram-positive bacteria with a high G+C content and are well known as prolific producers of biologically active secondary metabolites. Some genera were reported as producer of extracellular secondary metabolites that have larvicidal activity against mosquitoes (Rao *et al*., 1990). Microbial insecticides are being considered as alternative to chemical insecticides because of their selective toxicity and ready decomposability in the ecosystem. Also, unlike the inherent dangers associated with the process of production of synthetic insecticides, the process for the manufacture of microbial products is safe and less pollution (Misato, 1983).

*Corresponding author email: halamohamed6@yahoo.com

The present study has been undertaken to identify the actinomycete isolate which isolated from sand of underground spring in Egypt and showed a larvicidal activity against *Culex pipiens* to species level. Moreover, the efficiency of extracellular secondary metabolites of the actinomycete isolate under laboratory conditions was studied.

MATERIALS AND METHODS

Microorganism and culture conditions

During the isolation of mosquitocidal bacteria from sand samples of underground spring at Giza Governorate, Egypt, eight isolates of actinomycetes were isolated. One of them showed promising mosquitocidal activity against *Culex pipiens* and it was designated as Act-1. It was cultured on starch-nitrate agar medium (Küster and Williams, 1964) and incubated at 28°C.

Identification of Act-1

The cultural characteristics of Act-1 were studied on the basis of the International *Streptomyces* Project (ISP) recommended by Shirling and Gottlieb (1966). The morphological characters of this isolate were examined by the light and transmission electron microscopy (Tresner *et al*. 1961). The enzyme activities (proteolytic, lipolytic, lecithinase and pectinase) of Act-1were performed according to the method of Nitsch and Kutzner (1969) and Hankin *et al*. (1971). The utilization of nitrate and H_2S production were examined adopting the method of Williams *et al*. (1989). Arbutin decomposition was determined by the method of Kutzner (1976). Utilization of carbohydrates was investigated with a basal carbon nutrient medium (Pridham and Gottlieb, 1948). The antimicrobial activities of the culture broth of Act-1 were

examined by the agar diffusion method (Wu, 1984). The tested microorganisms were obtained from MIRCEN Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The cell wall composition including diaminopimelic acid (DAP) isomers and sugars were determined according to the methods of Hasegawa *et al*. (1983). Partial 16S rDNA analysis has been performed for the identification of Act-1 (De Soete, 1983 and Ludwig and Strunk, 1997).

Cultivation of actinomycete Act-1

A loopful of actinomycete isolate growth from starch-nitrate agar slop was transferred to 25 ml of growth medium in 250 ml Erlenmeyer conical flasks and incubated under static or shaking conditions at 28^{o}C for 5 days and harvested by centrifugation. The mycelial mass and the culture filtrate were bioassayed for toxicity against larva of *Culex pipiens.*

Effect of medium type

Five culture media of varying composition were tested for their ability to support the secondary metabolites production by Act-1 against larvae of *Culex pipiens* namely, (M1) peptone-beef extract-yeast extract-glucose medium (Greorgieva *et al*., 1966), (M2) has the same composition as M1 but without glucose, (M3) is the starch-nitrate medium, (M4) has the same composition of M3 but using glucose instead of starch and (M5) nutrient broth medium (Atlas, 1993).

Determination of the nutritional requirements for mosquitocidal metabolites production by Act-1 in M1 medium

One or more constituents of M1 medium were removed to study its/their effects on mosquitocidal metabolites production by Act-1.

Bioassay of mosquitocidal activity

Bioassay of mosquitocidal activity of Act-1 was detected according to the method of Ampofo (1995). Serial dilutions of the tested solution were prepared and the dilutions were placed into 100 ml beakers in triplicate along with *Culex pipiens* larvae. Uninoculated culture medium was served as control. The mortality percentage was recorded after 48 hours and corrected using appropriate control and adopting Abbott's formula (Abbott, 1925).

Statistical analysis

The data obtained statistically analyzed according to SPSS system using one-way analysis and the Duncan's multiple range test (Duncan, 1955) to determine the significance between means. Data were expressed as mean values ± standard errors. LC_{50} value was calculated by probit regression analysis (Proban version 1.1, Jedrychowski, 1991 shareware).

RESULTS AND DISCUSSION

The microscopic examination of Act-1 revealed that aerial mycelia were morphologically straight sporophore (Fig. 1). Each spore chain consisted of 10-20 turns with white and smooth surface (Fig. 2).

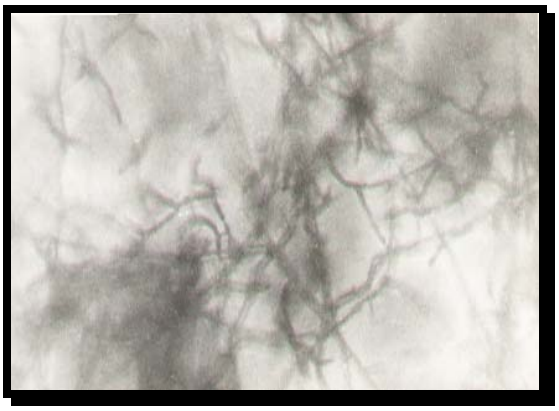

Fig. 1. Photograph showing spore chains of Act-1.

Fig. 2. Electron micrograph of spores of Act-1.

The cultural properties of Act-1 on various media are presented in Table 1. Act-1 grew well on most of the tested organic and synthetic media. The colonies were elevated, spreading and covered with white aerial mycelia and spores. Diffusable pigment was not produced.

The physiological characteristics of the tested isolate were listed in Table 2. Act-1 did not produce melanoid pigments on peptone-yeast extract-iron and tyrosine agars media. No proteolytic, lipolytic and lecithinase activity detected by Act-1 but moderate activity was showed for pectinase and arbutin degradation. Also Act-1 highly reduces nitrate with H_2S production. The utilization of various carbohydrates by Act-1 suggests a good pattern of carbon sources assimilation (Table 3). All of the sugars were utilized except inositol. Act-1 showed a narrow antimicrobial spectrum against the target microorganisms (Table 4).

Table 1. Culture characteristics of Act-1

Agar media used	Characteristics			
	Growth	Substrate mycelia	Aerial mycelia	Soluble pigment
Starch nitrate	Moderate	Yellow grey	White	-
Glycerol asparagine	Moderate	Light grey	White	-
Sucrose nitrate	Moderate	Slightly orange	White	-
Fish meal extract	Moderate	Medium brown	White	-
Soya bean meal	Moderate	Brown orange	White	-
Oat meal	Moderate	Light yellow brown	White	-
Malt-yeast extract	Moderate	Light yellow brown	White	-

Table 2. Physiological characteristics of Act-1

Reaction	Medium	Response
Melanin pigment production	PYI	-
	T	-
Enzyme activity		
Protyoltic	Egg-yolk	-
Lipolytic	Egg-yolk	-
Lecithinase	Egg-yolk	-
Pectinase	Hankin *et al*. (1971)	+
Nitrate reduction	Nitrate broth	+++
H_2S production	Nitrate broth	+
Degradation of arbutin	Kutzener *et al*. (1976)	+

PYI: peptone-yeast extract-iron medium, T: tyrosine medium, +++: good, +: moderate, -: negative

Analysis of the whole-cell hydrolysate of the tested isolate showed the presence of chemotype I cell wall characterized by LL-DAP acid. No diagnostic sugars were found.

Table 3. Utilization of carbohydrates by Act-1

Carbon sources	Response
No carbon	-
Glucose	+
Fructose	+
L-arabinose	+
Galactose	+
I-inositol	-
D-mannitol	+
L-rhamnose	++
Sucrose	++
D-xylose	+
Raffinose	+
Maltose	+

Trough morphological observations and chemotaxonomic characteristics, strain Act-1 could be characterized as belonging to the genus *Streptomyces*. In an attempt to identify it up to species level, a computerized database was used to compare the biological properties of Act-1 with those of other *Streptomyces* sp. Moreover, the partial sequence of Act-1 was applied to show the similarities to the closest relative in the 16S rDNA database. The obtained results showed that Act-1 has been enrolled into a cluster containing *Streptomyces microflavus*. By checking the similarity percentages, Act-1 is equidistant to *Streptomyces microflavus* (99.8%), based also to ABI database of partial and full sequence. The same equidistance to *Streptomyces microflavus* could be calculated using the MEDLINE database. These results suggest that Act-1 belong to *Streptomyces microflavus*. It was concluded that the use of genotypic and phenotypic techniques (polysporic approach) gives a better resolution in the species level identification (Stackebrandt and Wose, 1981).

Effect of Act-1 on *Culex pipiens* larvae

The clarified solution of Act-1 produced under static conditions was toxic to mosquito larvae of *Culex pipiens*. The precipitated part produced under both shaking and static conditions and clarified solution produced under shaking conditions did not show any activity against larva of *Culex pipiens*. Many workers reported that the secondary metabolites of actinomycetes produced by *Streptomyces aureus*, *Streptomyces avermitilis*, *Streptosporangium albidum*, *Streptomyces griseus*, *Micromonospora*, *Actinomandura*, *Actinoplanes*, *Micropolyspora*, *Nocardiopsis*, *Oerskonia*, *Thermomonospora*, *Streptoverticillium*, *Saccharopolyspora* and *Chainia* were toxic to mosquitoes (Ando, 1983; Zizka *et al*., 1989;

Anonymous, 1990; Rao *et al*., 1990 and Govindarajan *et al*., 2007).

Table 4. Antimicrobial activities of Act-1

Microorganisms	Zone of inhibition (mm)
Bacillus cereus	0
Bacillus subtilus	24
Escherichia coli	0
Candida albicans	0
Rhodotorula minuta	±
Debaryomces hansenii	±
Aspergillus niger	0
Asperagillus flavus	0
Asperagillus terrus	0
Trichoderma viride	0
Macrophomena phseoli	13
Fusarium vasenfectum	0
Botrytis alli	0
Alternaria humicola	13
Diplodia oryzae	20
Geotrichum candidum	0

Table 5. Effect of medium type on mosquitocidal activity of the metabolite of Act-1 against larvae of *Culex pipiens*

Medium type	Mortality % at 2000 ppm*
M1	95.4 ± 1.0 a
M2	78 ± 1.7 b
M3	31.7 ± .0.9 c
M4	20.8 ± 1.0 e
M5	27.5 ± 1.0 d

*Mortality % is presented as means ± SE. Values for each treatment followed by different letters are significantly different at $P = 0.05$.

Table 6. Effect of medium quantity on mosquitocidal activity of the metabolite of Act-1 against second instar larvae of *Culex pipiens*

Medium type/quantity (ml) of medium in 250 ml flask	Mortality % at 2000 ppm*
M1/	
25	97.5 ± 1.0 a
50	62.1 ± 1.6 b
75	13.8 ± 1.3 c
100	3.8 ± 0.7 d
M2/	
25	76.3 ± 2.1 a
50	62.9 ± 1.7 b
75	18.8 ± 0.9 c
100	2.9 ± 0.7 d

*Mortality % is presented as means ± SE. Values for each treatment followed by different letters are significantly different at $P = 0.05$.

Effect of culture media on production of secondary metabolites by Act-1

As shown in Table 5, M1 and M2 media were good media for production of mosquitocidal secondary metabolites of Act-1. The toxicity of the metabolites produced in M1 was 22% higher than that produced in M2 medium. M1 and M2 media contains peptone, beef extract and yeast extract which support the medium with a wide variety of amino acids, minerals and vitamins. M1 medium was enhanced the production of Act-1 secondary metabolites more than M2 medium because it contains glucose. Glucose was found to be an important nutrient for mycelium growth, secondary metabolites synthesis and control the fermentation of various antibiotics (Chen and Zhang, 2002). Increase of glucose concentration could promote the mycelium growth and spinosad production

Table 7. Effect of medium composition on mosquitocidal activity of the metabolite of Act-1 against second instar larvae of *Culex pipiens*

Medium composition	Mortality % at *		
	1000 ppm	500 ppm	100 ppm
1	83.8 ± 1.6 b	25.4 ± 1.3 d	4.2 ± 1.0 d
2	47.5 ± 1.7 e	13.8 ± 1.1 f	2.9 ± 0.7 de
3	100 ± 0.0 a	99.2 ± 0.6 a	27.5 ± 1.0 bc
4	100 ± 0.0 a	99.6 ± 0.4 a	25.4 ± 1.1 c
5	100 ± 0.0 a	98.8 ± 0.7 a	29.6 ± 1.3 b
6	75.4 ± 1.3 c	17.9 ± 1.3 e	2.9 ± 0.7 de
7	100 ± 0.0 a	33.3 ± 1.1 b	2.1 ± 0.7 de
8	100 ± 0.0 a	98.8 ± 0.7 a	50.4 ± 1.6 a
9	64.6 ± 1.1 d	30.0 ± 1.1 c	3.3 ± 0.7 d
Control (M1)	17.9 ± 0.7 f	1.3 ± 0.7 g	0.0 ± 0.0 e

*Mortality % is presented as means ± SE. Values for each treatment followed by different letters are significantly different at $P = 0.05$.

by *Saccharopolyspora spinosa* as reported by Zhihua *et al*. (2006).

Effect of medium quantity on mosquitocidal secondary metabolites produced by Act-1

Quantity of the medium is a critical factor for production of mosquitocidal secondary metabolites by Act-1 as shown in Table 6. The most favourable quantity of the medium for mosquitocidal secondary metabolites production was 25 ml in both media M1 and M2. Increasing the quantity of the medium, mosquitocidal secondary metabolites decreased gradually and disappeared at 100 ml. This indicated that 25 ml of the medium provides sufficient aeration to maximum mosquitocidal metabolites production by Act-1. As reported by Yengneswaran *et al*. (1991), the reduction in oxygen supply is an important limiting factor for growth and the secondary metabolites production of streptomycetes.

Effect of different combinations of M1 constituents on mosquitocidal metabolites production

A numbers of combinations of the constituents of M1 medium were tested to determine the effect of each constituent alone or in combination to other constituents on the mosquitocidal metabolites production by Act-1. As shown in Table 7, nine combinations enhanced the production of mosquitocidal secondary metabolites by Act-1. In all cases peptone, beef, yeast extracts and glucose were present in the medium (core). When $CaCO_3$ (1) or $MgSO_4$ (2) or K_2HPO_4 (3) or NaCl and $FeSO_4$ (4) added to the core, the produced toxicity was enhanced against *Culex pipiens* larvae. However, addition of $CaCO_3$ and NaCl (5) to the core enhanced the secondary metabolite production by Act-1 more than that of the first combination seven times. Addition of NaCl and $MgSO_4$ (6) or NaCl and K_2HPO_4 (7) or NaCl, $MgSO_4$ and $FeSO_4$ (9) to the core did not enhance the mosquitocidal metabolites production by tested organism. Addition of NaCl, $FeSO_4$ and $CaCO_3$ (8) to the core enhanced the produced mosquitocidal metabolites twelve times more than that of the first combination. This combination was found to be the most favourable combination for the metabolite production.

Streptomycetes are usually grown in complex media containing compounds such as peptone, beef or yeast extracts. These media are quite satisfactory for growth and production of secondary metabolites (Liu *et al*. 2008). Rapidly utilized sugars like glucose support increased growth rates at the expense of antibiotic production (Escalante and Ramos, 1999; Chen and Zhang, 2002; Zhihua *et al*., 2006). Increase in biomass production was not necessarily correlated with the increase of secondary metabolites production. Generally, a quickly metabolised substance like glucose is responsible for catabolite repression (Marwick *et al*., 1999).

Metal ions are essentially required for both growth and antibiotic formation, although their optimal concentrations for growth and antibiotic biosynthesis were different. It is known that NaCl added to the media of microorganisms enhance the availability of soluble protein in the medium (Morris *et al*., 1996). Maximum activity of antibiotic biosynthesis by *Streptomyces anulatus* was detected with Fe^{2+} and slightly decreased with Ca^{2+} (Marwick *et al*., 1999). Calcium was essentially required for both growth and antibiotic formation, although its optimal concentration for growth and antibiotic biosynthesis were different. Calcium was found to promote cellular growth **(**Morris *et al*., 1996).

In conclusion, the combinations of peptone, beef, yeast extracts, glucose, NaCl, $FeSO_4$ and $CaCO_3$ was the most suitable for mosquitocidal metabolites production by Act-1 with LC_{50} of about 77.3 ppm with 95% fiducial limits (62.6-95.7).

REFERENCES

Abbott, WS. 1925. A method of computing effectiveness of insecticide. Journal of Economical Entomology. 18:265-267.

Ampofo, JA. 1995. Use of local raw materials for the production of *Bacillus sphaericus* insecticides in Ghana. Biocontrol Science Technology. 5:417-423.

Ando, K. 1983. How to discover new antibiotics for insecticidal use. In: Pesticide chemistry: Human welfare and the environment, (vol. 2), Natural Products. Eds. Takahashi, T., Yoshioka, H., Misato, T. and Matusunaka, S. Pergman Press, New York. 253-259.

Anonymous, T. 1990. Biologically active KSB-1939L3 compound and its production. Pesticide with insecticide and acaricide activity production by *Streptomyces* sp. culture. Biotechnology Abstract 9 (19), 58. (Japan patent, no 273961, 1988).

Atlas, R.M. 1993. Hand book of microbiological media. Lawrence C. Parks, CRC Press, Boca Raton, Ann Arbor, London, Tokyo. 666-789.

Chen, JF. and Zhang, YX. 2002. Studies on the effect of phosphate concentration in sisomicin fermentation. Chinese Journal of Antibiotics. 27:452-455.

De Soete, G. 1983. A least square algorithm for fitting trees to proximity data. Psychometrica. 48:621-625.

Duncan, DB. 1955. Multiple range and multiple F-test. Biometrics. 11:1.

El-Kady, GA., Kamal, NH., Mosleh, YY. and Bahgat, IM. 2008. Comparative toxicity of two bio-insecticides (spinatoran and vertemic) compared with methomyl against *Culex pipiens* and *Anopheles multicolour*. World Journal of Agricultural Sciences. 4:198-205.

Escalante, I. and Ramos, I. 1999. Glucose repression of anthracyclin formation in *Streptomyces peucetius* var *caesius*. Applied Microbiology and Biotechnology. 52: 572-578.

Greorgieva, I., Sheikova, G. and Isov, P. 1966. Biosynthesis of antibiotic 255 under laboratory conditions. Antibiotiki. 11:1067-1079.

Grovindarajan, M., Jebanesan, A. and Reetha, D. 2007. Larvicidal efficacy of extracellular metabolites of actinomycetes against dengue vector mosquito *Aedes aegypti* Linn. (Diptera: Culicidae). Research and Reviews in Biosciences. 1:1-10.

Hankin, L., Zucker, M. and Sands, DC. 1971. Improved solid medium for the detection and enumeration of pectolytic bacteria. Applied Microbiology. 22:205-209.

Hasegawa, T., Takizawa, M. and Tanida, S. 1983. A rapid analysis for chemical grouping of aerobic actinomycetes. Journal of General and Applied Microbiology. 29:319-322.

Küster, E. and Williams, ST. 1964. Selection of media for isolation of streptomycetes. Nature. 202:928-929.

Kutzner, HJ. 1976. Methoden zur Untersuchung von Streptomyceten und einigen anderen Actinomyceten. Darmstadt: Teilsammlung Darmstadt am Institut für Mikrobiologie der Technischen Hochschule. TU Darmstadt. 1-154.

Liu, H., Qin, S., Wang, Y., Li, W. and Zhang, J. 2008. Insecticidal action of quinomycin A from *Streptomyces* sp. KN-0647, isolated from a forest soil. World Journal of Microbiology and Biotechnology. 24:2243-2248.

Ludwig, W. and Strunk, D. 1997. ARB-A software environment for sequence data. TU Munchen. 1-127 .

Marwick, JD., Wright, PC. and Burgess, TG. 1999. Bioprocess intensification for production of novel marine bacterial antibiotics through bioreactor operation and design. Biotechnology. 1:495-507.

Misato, T. 1983. Recent status and future aspects of agricultural antibiotics. In: Pesticides chemistry: Human welfare and Environment, (vol. 2). Natural Products. Pergman Press. Oxford. 241-246.

Morris, ON., Converse, V., Kanagaratnam, P. and Davies, JS. 1996. Effect of cultural conditions on spore-crystal yield and toxicity of *Bacillus thuringiensis* subsp. a*izawai* (HD 133). Journal of Invertebrate Pathology. 67:129-136.

Nitsch, B., and Kutzner, HJ. 1969. Egg-yolk as a diagnostic medium for streptomycetes. Experientia. 25:113.

Peng, Z., Yang, J., Wang, H. and Simons, FER. 1999. Production and characterization of monoclonal antibodies to two new mosquito *Aedes aegypti* salivary proteins. Insect Biochemistry and Molecular Biology. 29:909-914.

Pridham, TG. and Gottlieb, D. 1948. The utilization of carbon compounds by some actinomycetes as an aid for species determination. Journal of Bacteriology. 56:107-114.

Rao, KV., Chattopadhyay, SK. and Reddy, GC. 1990. Flavonoids with mosquito larval toxicity. Tangeration, daidzein and genistein crystal production, isolation, and purification, *Streptomyces* spp culture, insecticide. Journal of Agriculture and Food Chemistry. 38:1427-1430.

Service, MW. 1983. Management of vectors. In: Pest and Vectors Management in Tropics. Eds. Youdeowei, A. and Service, MW. Longman Group Ltd., England. 7-20.

Shirling, EB. and Gottlieb, D. 1966. Methods for characterization of *Streptomyces* species. International Journal of Systematic Bacteriology. 16:313-340.

Stackebrandt, E. and Woese, C. R. 1981. Towards a phylogeny of actinomycetes and related organisms. Current Microbiology. 5:197.

Tresner, HD., Davies, MC. and Backus, EJ. 1961. Electron microscopy of *Streptomyces* spore morphology and its role in species differentiation. Journal of Bacteriology. 81:70-80.

Vijayan, V., and Balaraman, K. 1991. Metabolites of fungi and actinomycetes active against mosquito larvae. Industrial Journal of Medical Research. 93:115-117.

Williams, S.T., Sharp, M.E. and Holt, J.G. 1989. Bergey's Manual of Determinative Bacteriology (vol. 4). The Williams and Wilkins. Co. Baltimore, London.

Wu, RY. 1984. Studies on the *Streptomyces* SC4. II Taxonomic and biological characteristics of *Streptomyces* strain SC4. Botanica Bulltein Academic Sinica 25:111-123.

Yengneswaran, PK., Gray, MR. and Thompson, BG. 1991. Effect of dissolved oxygen control on growth and antibiotic production in *Streptomyces clavuligerus* fermentations. Biotechnology Progress. 7:246-250.

Zhihua, J., Xiu, C. and Peilin, C. 2006. Effects of glucose and phosphate on spinosad fermentation by *Saccharopolyspora spinosa*. Chinese Journal of Chemical Engineering. 14:542-546.

Zizka, Z., Weiser, J., Blumauerova, M. and Jizba, J. 1989. Ultra structural effects of macroterrolides of *Streptomyces grieseus* LKS-1 in tissues of *Culex pipiens* larvae-monactin, dinactin, triactin and nonactin preparations, insecticide activity. Cytobios. 58:85-91.

Received: Oct 1, 2009; Accepted: Nov 11, 2009