International Journal of Microbiology Research
ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 5, Issue 3, 2013, pp.-399-403.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000234

MEDIUM OPTIMIZATION OF *Burkholderia gladioli pv. agaricicola* ENHANCED THE PRODUCTION OF ANTIMICROBIAL SUBSTANCES

ELSHAFIE H.S.[1], CAMELE I.[1]* AND BUFO S.A.[2]

[1]School of Agricultural, Forestry, Food and Environmental Sciences, University of Basilicata, Viale dell'Ateneo Lucano, 10, 85100 Potenza, Italy.
[2]Department of Science, University of Basilicata, Via dell'Ateneo Lucano, 10, 85100 Potenza, Italy.
*Corresponding Author: Email- ippolito.camele@unibas.it

Received: March 01, 2013; Accepted: March 28, 2013

Abstract- Numerous species in the genus *Burkholderia* have interesting properties for potential industrial applications including production of antibiotics, biosurfactants, bioplastics and degradation of environmental contaminants. The aim of this research was study the effect of different carbon and nitrogen sources on *Burkholderia gladioli* pv. *agaricicola* strain ICMP11096 growth and bioactivity of produced secondary metabolites against gram-positive bacterium *Bacillus megaterium* and plant pathogenic fungi *Rhizoctonia solani*. The additional carbon sources were sucrose, fructose and lactose whereas, the additional nitrogen sources were, urea, potassium nitrate and ammonium nitrate. The results demonstrated that the addition of new carbon and nitrogen sources to the minimal mineral nutrient media lead to decreasing the growth rate of studied bacterial strain and increasing the production of bioactive substances. The maximum production of bioactive substances of studied bacterial strain was obtained using ammonium nitrate and lactose. The purified filtrate of the studied bacterial strain was fractionated by High Performance Liquid Chromatography (HPLC) and the antimicrobial activity of five isolated single peaks was evaluated against gram positive bacteria *B. megaterium* ITM100 and gram negative bacteria *Escherichia coli* ITM103. The most bioactive peak was number two with 12800 and 6400 Ua.ml^{-1} against *B. megaterium* and *E. coli*, respectively. The obtained results suggested that the nutrient amendments can increase the production of antimicrobial substances and this may be a useful strategy for improving the biocontrol efficiency.

Keywords- Antimicrobial activity, Secondary metabolites, Carbon sources, Nitrogen sources, Bacterial growth rate.

Citation: Elshafie H.S., Camele I. and Bufo S.A. (2013) Medium Optimization of *Burkholderia gladioli pv. agaricicola* Enhanced the Production of Antimicrobial Substances. International Journal of Microbiology Research, ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 5, Issue 3, pp.-399-403.

Introduction

Increasing the environmental concerns of production the bioactive microbial substances for application as biopesticides are considered promising alternatives to synthetic pesticides. There are some important factors influencing the bacterial growth including physical requirements and available sources of chemical nutrients [1].

The production of bacterial secondary metabolite depend, also, on the nutrient status of culture medium [2,3] and is related with some various aspects of gene regulations and environmental factors [4,5]. The minimal mineral agar (MMA) is considered the best nutrient media suitable for study the growth of *Burkholderia* sp. [6] because it contains the minimum nutrients possible for bacterial growth. A defined medium has known quantities of all ingredients to provide a defined carbon and nitrogen source as well as essential trace elements and vitamins required by the microbe [7]. Carbon is the structural backbone of the organic compounds that make up a living cell [8] such as: glucose, galactose, sucrose and starch. Nitrogen is needed for the synthesis of such molecules as amino acids, DNA, RNA and ATP [5].

Demain [9], observed that the biosynthesis of the microbial secondary metabolites is induced by the addition of a nutrition system and by decreasing the growth rate so that the bacterial cells could reproduce slowly and reach to the maximum colony form unit (CFU) in longer period and the bioactivity of produced metabolites increased gradually to reach the maximum value during the stationary phase.

N.Acyl Homoserine lactones (*N*.AHLs) signal molecule based quorum sensing (QS) phenomenon play a vital regulatory role in chemical differentiation of secondary metabolism and morphological differentiation of morphogenesis which is directly proportional with the CFU [9-12]. Recent investigations have shown that *Burkholderia gladioli* pv. *agaricicola* (*Bga*) produces the above signal molecule [13] and have confirmed the production of group secondary metabolites, which could include novel active compounds [14]. Some strains of *Bga* have showed antifungal activity against some serious phytopathogenic fungi [15]. The aims of the present research were: (i) study the effect of different carbon and nitrogen sources on *Bga* ICMP11096 growth and increasing the production of its bioactive secondary metabolites, (ii) study the bioactivity of produced metabolites in modified substrates against gram-positive bacterium *Bacillus megaterium* (*B.meg*) and plant pathogenic fungi *Rhizoctonia solani* (*R.sol*), (iii) HPLC fractionation of produced secondary metabolites of *Bga* ICMP11096 and evaluate the bioactivity of isolated peaks against *B.meg* and *Escherichia coli* ITM103 (*E.col*).

Materials and Methods

Bacterial and Fungal Strains

The wild type strain of *Bga* ICMP11096 was isolated from *Agaricus bitorquis* (Quelet) Saccardo and obtained from ICMP Collection of Landcare Research (Auckland, New Zealand). This strain was kept as pure-freeze lyophilized stock maintained collection of School of Agricultural, Forestry, Food and Environmental Sciences, Basilicata University (Potenza, Italy). The studied target phytopathogenic fungus was *R.sol* whereas the target bacterial strain was gram positive *B.meg* ITM100 and *E.col* ITM103. The tested fungi and bacteria strains were derived from a pure culture maintained collection of the same department kept on potato dextrose agar (PDA) and King B (KB) respectively at 8°C.

Preparation of Modified Media

Three different carbon and nitrogen sources were supplemented separately to the MM liquid broth media as following; sucrose 5 $g.l^{-1}$, fructose 5.2 $g.l^{-1}$ and lactose 5.2 $g.l^{-1}$ are containing 0.175 mols of carbon atoms and urea 3.0 $g.l^{-1}$, potassium nitrate 10.1 $g.l^{-1}$ and ammonium nitrate 4.0 $g.l^{-1}$ are containing 0.1 mols nitrogen atoms. Erlenmeyer flasks containing 25ml of nutrient MM broth media were inoculated with 1 ml suspension of *Bga* ICMP11096 containing about 10^8 $CFU.ml^{-1}$ (optical density, O.D. 0.2 at 590 nm) from fresh culture of 24h age, and were incubated at 25°C under shaking (180 rpm). Three replicates were prepared for each nutrient treatment. The additional cultures were prepared as control composed only from MM plus each carbon and nitrogen sources.

Effect of Carbon and Nitrogen Sources on the Filtrates Bioactivity

The preparation of bacterial filtrates was carried out by centrifugation of broth culture at 20.000 *g* for 15 min and filtration using sterile Millipore filter 0.22 mm. For the antifungal test, *R.sol* was grown on PDA medium and agar well diffusion method was carried out using the method of Magaldi *et al.* [16] as following: wells of 0.5 cm diameter were punched into the nutrient medium and filled with 10 µl of prepared cell-free culture filtrates using serial dilutions (1:1 v/v). The plates were left for 24 h in a refrigerator at 4°C for completely diffusion. The plates were then incubated at 25°C for 96h. In case of antibacterial test, *B.meg* was grown on KB medium and the test was carried out using the method of Lavermicocca *et al.* [17] as following: 10 µl using serial dilutions (1:1 v/v) of cell-free culture filtrates were dropped on 10 ml of solid KB (in a 90x10 mm Petri dish) [18]. Completely dryness, 4 ml of soft agar (0.7 %) containing 0.5 ml of bacterial target suspension of 10^8 $CFU.ml^{-1}$ of *B.meg* was poured on the plate surface and then the plates were incubated at 25°C for 48h. The activities were expressed as Unit active for millilitre ($Ua.ml^{-1}$) where unit is the amount of substance contained in 10 ml capable of inhibiting the growth of the target organism [19].

Effect of Carbon and Nitrogen Sources on Bacterial Growth

The bacterial growth was measured for each sample using spectrophotometer UNICAM-UV 500 apparatus at 600 nm to determine the optical density that refer to the bacterial growth in each media.

Purification and Extraction of Diffusible Secondary Metabolites

The purified filtrate of the most bioactive *Bga* strain ICMP11096 was analysed by High Performance Liquid Chromatography (HPLC). The analysis involved two main stages: sample preparation and fractionation by HPLC.

Sample Preparation by Solid Phase Extraction

Aliquots (30 $mg.ml^{-1}$) of lyophilized bioactive 5 days old culture filtrates of wild type *Bga* strain ICMP11096 was taken up in solution in sterile distilled water and the resulting solution loaded on cartridge syringe (Strata C18-T) prewashed with 2 ml methanol followed by 2 ml distilled water. The loaded solution was 10 ml containing 300 mg of lyophilized substances. After that, the cartridge was washed with 1 ml distilled water and recovered by 1 ml of methanol that was collected in sterilized eppendorf.

HPLC Fractionation

The methanol fraction was analyzed in the analytical HPLC-Agilent 1200 series austere. The best separation was obtained with an Agilent ECLIPSE XDB, C18 (4.6 x 150 mm, 5 µm) in analytical conditions. The injected volume was 20 µl, column temperature was 25°C, autosampler chamber temperature was 4°C, flow rate was s 1.0 $ml.min^{-1}$ and mobile phase were: A: 0.2% Formic acid (FA) in H_2O and B: 0.1% FA in Acetonitrile (CH_3CN). The HPLC chromatogram was obtained at wave length l= 380 nm. The antimicrobial activity of isolated single peaks was evaluated against *B.meg* ITM100 and *E.col* ITM103 as reported before by Lavermicocca *et al.,* [17] and the bioactivity was expressed as $Ua.ml^{-1}$ [19].

Results and Discussion

Effect of Carbon and Nitrogen Sources on the Filtrates Bioactivity

The maximum antifungal activity against *R.sol* was observed using ammonium nitrate with lactose and fructose in nutrient media. The activity was 3200 $Ua.ml^{-1}$ after 13 days of incubation [Table-1]. The increase of bioactive substances was observed between 3-7 days using ammonium nitrate with fructose and lactose and reached a maximum after 13 days compared to control. These results are considered the positive effect for using ammonium nitrate as nitrogen source with all studied carbon sources.

Table 1- Antifungal activity of Burkholderia gladioli pv. agaricicola strain ICMP11096 against Rhizoctonia solani.

Nutrient Media	Bioactivity Expressed as $Ua.ml^{-1}$ Incubation Period (Day)				
	3	7	9	13	16
Control (MM)	200	400	800	200	0
N1+C1	200	400	800	200	0
N1+C2	100	400	800	200	0
N1+C3	100	400	800	200	0
N2+C1	400	800	1600	400	0
N2+C2	200	400	800	1600	200
N2+C3	200	400	800	1600	200
N3+C1	400	800	1600	2400	400
N3+C2	400	800	1600	400	100
N3+C3	800	1600	2400	3200	400

C1: Sucrose, C2: Lactose, C3: Fructose, N1: Urea, N2: Potassium nitrate and N3: Ammonium nitrate.

The maximum antibacterial activity against *B.meg* was observed using ammonium nitrate with lactose and fructose. The activity was 6400 $Ua.ml^{-1}$ after 13 days of incubation [Table-2]. The increase of bioactive substances was observed between 3-7 days and reached a maximum after 13 days compared to control. The obtained results of antimicrobial activity confirmed the hypothesis that the addition of carbon sources in the beginning of life cycle of bacteria plays an essential role in prolonging the period of production of the bioactive substances due to the best adaptation of tested bacteria to the nutrient media during the log phase than stationary phase and hence

the net production of the total active substances were maximized at the end of stationary phase in accordance with Hengge-Aronis and Lazazzera [20,21] who reported that the transition into stationary phase is directed by some key transcription factors which are stimulated by starvation.

Table 2- Antibacterial activity of Burkholderia gladioli pv. agaricicola strain ICMP11096 against Bacillus megaterium.

Nutrient Media	Bioactivity Expressed as Ua.ml^{-1} Incubation Periad (day)				
	3	7	9	13	16
Control (MM)	200	1600	400	200	0
N1+C1	200	1600	400	200	0
N1+C2	200	800	200	0	0
N1+C3	200	800	1600	400	0
N2+C1	400	1600	3200	200	0
N2+C2	200	800	1600	400	100
N2+C3	400	1600	3200	400	0
N3+C1	400	1600	3200	800	100
N3+C2	200	800	1600	400	100
N3+C3	400	2400	3200	6400	400

C1: Sucrose, C2: Lactose, C3: Fructose, N1: Urea, N2: Potassium nitrate and N3: Ammonium nitrate.

Effect of Carbon and Nitrogen Sources on the Bacterial Growth

The mid log phase in control media started faster than using urea with all tested carbon sources where the maximum growth achieved after 9 days of incubation [Fig-1]. Using potassium nitrate leads to prolong the log phase compared to control media especially with lactose and fructose due to the reduction of growth rate and the maximum growth was achieved after 13 days in case of potassium nitrate with fructose [Fig-2]. Using ammonium nitrate leads to prolonging the log phase compared to control media indicating the highly adaptation of bacteria toward the new nutrient components especially with lactose, fructose and sucrose respectively. The maximum growth using ammonium nitrate and lactose was achieved after 13 days [Fig-3].

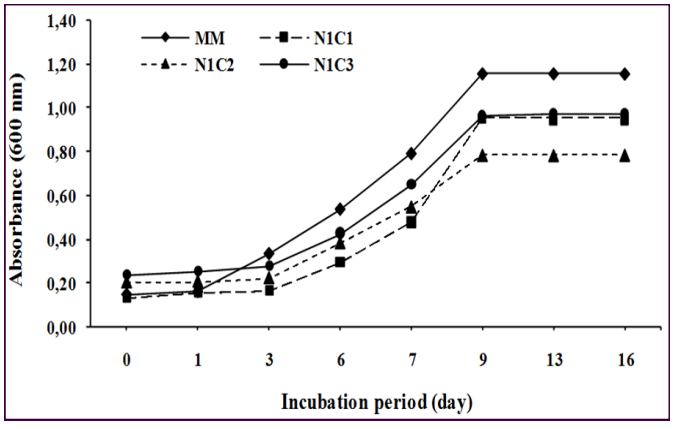

Fig. 1- Bacterial growth curve of *Burkholderia gladioli* pv. *agaricicola* ICMP11096 with N1 (C1, C2, C3).

C1: Sucrose, C2: Lactose, C3: Fructose and N1: Urea.

The obtained results of optimization the nutrient media could investigate the vital regulatory role of *N*.AHLs signal molecule in the production of secondary metabolites. This signal molecule can be produced with high amount at the maximum bacterial cell density to induce the high production of secondary metabolites with maximum bioactivity [22,23]. The nutrient media components effect on the expression of quorum sensing phenomenon which plays a central role in the gene regulation of specific bacterium to encode the more efficient functions at high cell density such as production of secondary metabolites [24,25]. The above hypothesis is confirmed by the low molecular weight nature of the signal molecules for inactivating the regulatory protein to decrease the production of secondary metabolites and enhance the morphogenesis during the rapid growth rate. So the decrease of bacterial growth rate induces the biosynthesis of secondary metabolites and reaches to the maximum production in parallel with the formation of signal molecules at high cell density in agreement with the results obtained by Demain [9].

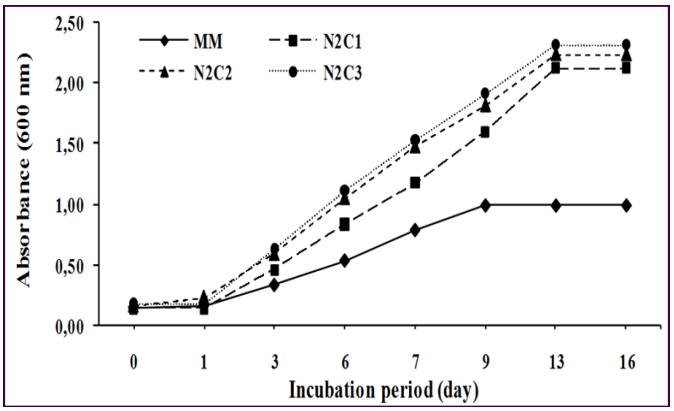

Fig. 2- Bacterial growth curve of *Burkholderia gladioli* pv. *agaricicola* ICMP11096 with N2 (C1, C2, C3).

C1: Sucrose, C2: Lactose, C3: Fructose and N2: Potassium nitrate.

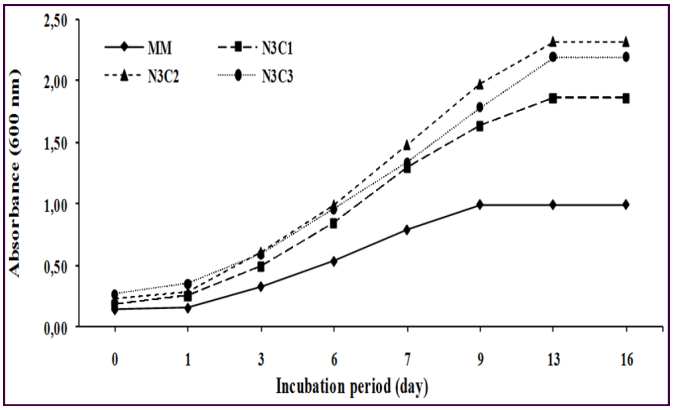

Fig. 3- Bacterial growth curve of *Burkholderia gladioli pv. agaricicola* ICMP11096 with N3 (C1, C2, C3).

C1: Sucrose, C2: Lactose, C3: Fructose and N3: Ammonium nitrate.

HPLC Chromatogram of Cell-Free Culture Filtrate of *Burkholderia gladioli* pv. *agaricicola* ICMP11096

From chromatographic runs conducted at l = 380 nm, it was noticed the presence of five single peaks [Fig-4]. The five peaks were collected and evaluated for their antimicrobial activity against *B.meg* and *E.col*. In particular; the most bioactive peaks were number 2 and 3 [Table-3], [Fig-5]. The identification of bioactive peaks is in progress.

Table 3- Antimicrobial activity of single HPLC peaks

Peaks	Antimicrobial activity Ua.ml^{-1}	
	Bacillus megaterium	*Escherichia coli*
1	3200	1600
2	12800	6400
3	6400	3200
4	1600	800
5	3200	1600

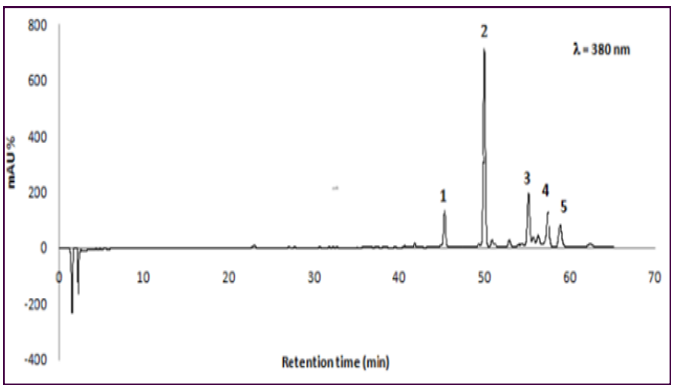

Fig. 4- HPLC chromatogram of ICMP11096 of *Burkolderia gladioli* pv. *agaricicola* at 380 nm.

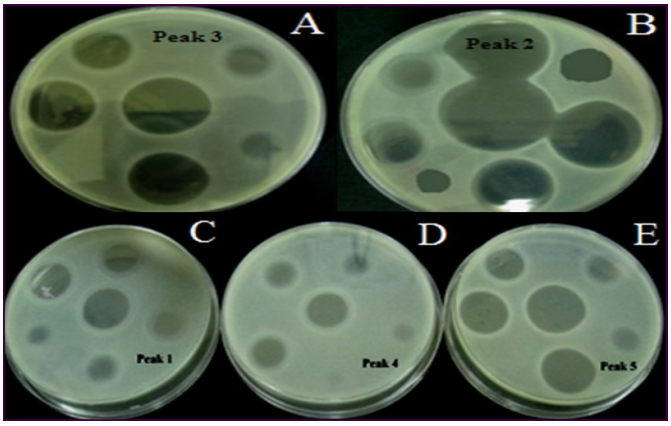

Fig. 5- Antimicrobial activity of single HPLC peaks vs. *Bacillus megaterium.* Where: A: Peak 3, B: Peak 2, C: Peak 1, D: Peak 4 and E: Peak 5.

Conclusion

Results obtained in this study suggested that the nutrient amendments may increase the production of antimicrobial substances and this may be a useful strategy for improving the biocontrol efficiency in accordance with Duffy and Defago [26]. Moreover, the addition of new carbon and nitrogen sources at the beginning of lag phase is useful to prolong the transition into stationary phase due to decreasing the competition between bacterial colonies and lowering the growth rate of bacterial cells which resulting in highly production of bioactive substances as well. On the other hand, the addition of those amendments during the mid log phase causes a highly competition and increases the growth rate and decreasing the production of antimicrobial substances. In fact, the bacterial colonies must adapted themselves to the nutrient media components during the lag phase for inducing the specific genes for production the antimicrobial substances and reach to the maximum growth at the end of exponential phase in order to enter the stationary phase and produce the highest quantity of bioactive substances [27,28]. Generally, in the present study, it was concluded that the maximum antimicrobial activity of *Bga* ICMP11096 was obtained after incubation for 13 days at 25°C in MM nutrient media adjusted at 7.0 pH and supplemented with ammonium nitrate 4 $g.l^{-1}$ and lactose 5.2 $g.l^{-1}$.

Acknowledgments

We are grateful to Dr. Parisien Michèle Lynn Marie (University Language Centre-UNIBAS-CLA) for English revision and thanks for the technical assistance from C.T. Michele Palumbo.

Conflict of Interest

The authors declare no conflict of interest

List of Used Abbreviations along the Manuscript

Bacillus megaterium : *B.meg*

Burkholderia gladioli pv. *agaricicola* : *Bga*

Colony form unit : CFU

Escherichia coli : *E.col*

High Performance Liquid Chromatography : HPLC

International Collection of Microorganisms from Plants : ICMP

King B : KB

Minimal Mineral Agar : MMA

*N.*Acyl Homoserine lactones : *N.*AHLs

Potato Dextrose Agar : PDA

Quorum sensing : QS

Rhizoctonia solani : *R.sol*

Unit active of millilitre : $Ua.ml^{-1}$

References

[1] Prapagdee B., Kuekulvong C. and Mongkolsuk S. (2008) *Int. J. Biol. Sci.,* 4 (5), 330-337.

[2] El-Banna N. and Winkelmann G. (1998) *J. Appl. Microbiol.,* 85, 69-78.

[3] Hwang J., Chilton W.S. and Benson D.M. (2002) *Biol. Control,* 25, 56-63.

[4] Duffy B.K. and Defago G. (2000) *Appl. Environ. Microbiol.,* 66, 3142-3150.

[5] Young S.K., Lee Y.J., Lee Y.H. and Kim J.H. (2009) *J. Microbiol. Biotechnol.,* 19(10), 1142-1149.

[6] Howard K. and Inglis T.J. (2005) *Water Res.,* 39(6), 1085-1092.

[7] Madigan M. and Martinko J. (2005) *Brock Biology of Microorganisms*. San Francisco, CA: Benjamin.

[8] Fagerbakke K.M., Heldal M. and Norland S. (1996) *Aqatic Microb. Ecol.,* 10, 15-27.

[9] Demain A.L. (1998) *Int. Microbiol.,* 1, 259-264.

[10] Withers H., Swift S. and Williams P. (2004) *Curr. Opin. Microbiol*., 4, 186-193.

[11] Liu X., Bimerew M., Ma Y., Muller H., Ovardis M., Eberl L., Berg G. and Chernin L. (2007) *FEMS Microbiol. Lett.,* 270, 299-305.

[12] Duerkrop B.A., Ulrich R.L. and Greenberg E.P. (2007) *J. Bacteriol.,* 189, 5034-5040.

[13] Prashanth S.N., Bianco G., Cataldi T.R. and Iacobellis N.S. (2011) *J. Agric. Food Chem.,* 59, 11461–11472.

[14] Lamorte D., Elshafie H.S., Lelario F., Bufo S.A. and Iacobellis N.S. (2010) 12*th Symposium on Chemistry and Fate of Modern Pesticides*, Matera- Italy. 350-351.

[15] Elshafie H.S., Camele I., Racioppi R., Scrano L., Iacobellis N.S. and Bufo S.A. (2012) *Int. J. Mol. Sci.* 13, 16291-16302.

[16] Magaldi S., Mata-Essayag S., Hartung de Capriles C., Perez C., Colella M.T., Olaizola C. and Ontiveros Y. (2004) *Int. J. Infect. Dis*., 8, 39-45.

[17] Lavermicocca P., Iacobellis N.S., Simmaco M. and Graniti A. (1997) *Physiol. Mol. Plant Pathol*., 50, 129-140.

[18]King E.O., Ward M.K. and Raney D.E. (1954) *J. Lab. Clin. Med.,* 44, 301-307.

[19]Sinden S.L., DeVay J.E. and Backman P.A. (1971) *Physiol. Plant Pathol.,* 1, 199-213.

[20]Hengge-Aronis R. (1999) *Curr. Opin. Microbiol.,* 2, 158-152.

[21]Lazazzera B.A. (2000) *Curr. Opin. Microbiol.,* 3, 177-182.

[22]Lazazzera B.A., Palmer T., Quisel J. and Grossman A.D. (1999) *Cell-Cell Communication in Bacteria*, ASM Press, 27-46.

[23]Seo Y.S., Lim J., Choi B.S., Kim H., Goo E., Lee B., Lim J.S., Choi I.Y., Moon J.S., Kim J. and Hwang I. (2011) *J. Bacteriol.,* 193, 3149-3149.

[24]Velusamy P. and Gnanamanickam S.S. (2008) *Secondary Metabolites in Soil Ecology*, Springer-Verlag Berlin Heidelberg, 5.

[25]Elshafie H.S., Iacobellis N.S. and Bufo S.A. (2012) *Fifth Saudi Science Conference* (*SSC5*), 92-93.

[26]Duffy B.K. and Defago G. (1999) *Appl. Environ. Microbiol.,* 65, 2429-2438.

[27]You Z., Fukushima J., Tanaka K., Kawamoto S. and Okuda K. (1998) *FEMS Microbiol. Lett.,* 164, 99-106.

[28]Steindler L. and Venturi V. (2007) *FEMS Microbiol. Lett.,* 266, 1-9.