African Journal of Biotechnology

Volume 15 Number 26, 29 June 2016

ISSN 1684-5315

Academic Journals

ABOUT AJB

The African Journal of Biotechnology (AJB) (ISSN 1684-5315) is published weekly (one volume per year) by Academic Journals.

African Journal of Biotechnology (AJB), a new broad-based journal, is an open access journal that was founded on two key tenets: To publish the most exciting research in all areas of applied biochemistry, industrial microbiology, molecular biology, genomics and proteomics, food and agricultural technologies, and metabolic engineering. Secondly, to provide the most rapid turn-around time possible for reviewing and publishing, and to disseminate the articles freely for teaching and reference purposes. All articles published in AJB are peer-reviewed.

Contact Us

Editorial Office: ajb@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJB

Submit manuscript online http://ms.academicjournals.me/

African Journal of Biotechnology

Table of Content: Volume 15 Number 26 29 June, 2016

ARTICLES

academicJournals

Vol. 15(26), pp. 1383-1390, 29 June, 2016
DOI: 10.5897/AJB2014.14353
Article Number: 992DB3B59223
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Ameliorative effect of *Lentinus squarrosulus* mycomeat against *Pseudomonas aeruginosa* infection using albino rat as animal model

Bamigboye C. O.* and Oloke J. K.

Department of Pure and Applied Biology (Microbiology Unit), Ladoke Akintola University of Technology, P.M.B 4000, Ogbomoso, Oyo State, Nigeria.

Received 4 December, 2014; Accepted 13 May, 2016

The increasing awareness of inherent therapeutic and prophylactic benefits of some higher fungi and their products has been the recent trend for improving a healthy vigour. Mycomeat is a mushroom derived 'meat-like' product produced using solid state fermentation technique. Previous research in this group revealed mycomeat as a potentially useful neutraceutical. This study seeks to explore the potential of mycomeat as a therapeutic agent against *Pseudomonas aeruginosa* infections. Three concentrations (83.3, 166.6 and 250 mg/kg body weight) of powdered mycomeat were prepared and administered to *P. aeruginosa* infected wistar rats. Morphological appearance and behavior of the rats were used as the assessment method for adverse reactions. After a period of 26 days, the rats were sacrificed with the liver, spleen and testes aseptically excised for histopathological analysis, the sperm count and sperm motility assay was carried out. Histopathological analysis revealed the testis of therapeutic group administered with 83.3 mg/kg as having a normal seminiferous tubule, while other organs were adversely affected. The group administered with 166 mg/kg of mycomeat has a normal hepatocyte, normal seminiferous tubule and splenic cells. Results from the therapeutic group were consistently better than the prophylactic group. The untreated group showed anomaly in the three organs inspected. The sperm count but not motility was significantly higher in the treated group than that observed in the positive and negative control group. This study was able to establish that mycomeat could be used to preserve body organs, at a dose of 166 mg/kg body weight. It can also be used as a birth control neutracetical for males.

Key words: Mushroom, *Lentinus subnudus*, histopathology, birth control, fertility.

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous gram-negative bacterium that is virulent towards a wide range of organisms, including bacteria, plants, insects, and mammals (Plotnikova et al., 2000). *P. aeruginosa* is

*Corresponding author. E-mail: kemmai_me@yahoo.com. Tel: +2347031068220.

increasingly recognized as an emerging opportunistic pathogen of clinical relevance. In humans, *P. aeruginosa* chronically infects the lungs of most cystic fibrosis patients, causing serious infections of burn wounds and eye lesions, and systemic infections in immuno-compromised individuals (Holder, 1993). The bacterium's pathogenicity is displayed via its secreted and surface-associated virulence factors and the complexity of the regulatory unit controlling these factors. *P. aeruginosa* is also known to be resistant to most antibiotics. George et al. (1989) established that the oral dose of *P. aeruginosa* required to establish colonization in a healthy subject is high but not impossible. The study by Mena and Gerba (2009) showed that the risk of colonization from ingesting *P. aeruginosa* in drinking water is low and slightly higher if the subject is taking an antibiotic resisted by *P. aeruginosa*. Its occurrence in drinking water is related to its ability to colonize biofilms in faucets, showerheads etc. *P. aeruginosa* could cause pneumonia, urinary tract infections, gastrointestinal infections and endocarditis. Although, its mode of acquisition is still much controversial, Boyer et al. (2011) reported that contaminated tap water could be associated with *P. aeruginosa* acquisition. The colonization of the respiratory tract could be exogenous or endogenous such as from the intestine, the oropharynx and the gastric compartment (Forestier et al., 2008).

The liquid mycelia extract of *Lentinus squarrosulus* is known to have ulcer prevention and healing capabilities in rats, this establishes that this mushroom species is not only edible and nutritious but also possess tonic and medicinal qualities (Omar et al., 2011). Traditionally, mushrooms are used for nutritional, medicinal and mythological benefits in Nigeria (Osemwegie et al., 2006). Globally, the level of mushroom nutraceuticals established mushrooms as good health food and reports abound on their use for the treatment of malnutrition in infants, diabetes, obesity or hyperlipidemea, sterility, anemia, mumps, fever and protein deficiency (Idu et al., 2007). The presence of polysaccharides in mushrooms could make them useful as natural health promoters against parasites, bacteria and viruses (Oei, 2003). Both fruiting body and the mycelium of mushrooms contain compounds with wide ranging antimicrobial activities. They are rich sources of natural antibiotics, where the cell wall glucans are well known for their immunomodulatory properties, and many of the externalized secondary metabolites combat bacteria, fungi, and viruses (Collins and Ng, 1997). Our previous study on mycomeat production using *Lentinus squarrosulus* revealed it is nutritious and could be harnessed as a useful neutraceutical. However, reports on mycomeat generally are very few and to the best of our knowledge, no report on its therapeutic efficacy has been reported till date. This research therefore seeks to establish the *in-vivo* efficacy of mycomeat against *P. aeruginosa* infection in animal model.

MATERIALS AND METHODS

Mycomeat production

Mycomeat was prepared as described in our previous study (Bamigboye et al., 2013). Briefly, *L. squarrosulus* was cultured on soymilk residue at 25°C untill full ramification was obtained. The mycomeat produced was harvested, oven dried and manually milled.

Bacterial suspension

Pathogenic *P. aeruginosa* from the ear swab of a hospitalized patient was obtained from Bowen University Teaching Hospital, Ogbomoso, Oyo State, Nigeria and sub-cultured as research demands.

In-vivo antimicrobial assay

All the applicable guidelines for the care and use of animals were followed in the study. Seven days prophylactic and 1 h post infection effects of mycomeat on *P. aeruginosa* were investigated using the method previously described by Oke (2006).

Seven days prophylactic experiment

Eighteen, 16-weeks old albino rats were fed on pellets and sterile water *ad libitum*. They were divided into three groups, Set A_1, A_2 and A_3 administered with 83.3 mg/kg bodyweight of mycomeat, 166.6 mg/kg bodyweight and 250 mg/kg bodyweight, respectively. Seven days post mycomeat administration, an aliquot of (4.0×10^7 ml) bacterial suspension was galvaged into each animal. The rats were observed for 26 days, after which they were sacrificed by cervical dislocation. The liver, spleen and testes were excised for histopathological analysis, sperm motility and sperm count was also analysed.

One-hour post bacterial administration/ therapeutic experiment

Aliquot of (4.0×10^7/ ml) bacterial suspension was administered orally into each of twenty-four animals. After 1 h, they were divided into four groups of 6 each, Set B_1, B_2 and B_3 were treated as above. The control group was administered with 0.2 ml of (4.0×10^7/ ml) bacterial suspension alone without treatment (Cp). Other control groups were administered with mycomeat suspension alone (Cm) and the last group was given feed and water only (C_{01}). The rats were followed for 26 days, after which they were sacrificed by cervical dislocation. Post- infection assay was done by using the method of Majolagbe et al. (2013) to establish the colonization of the test organism.

Histological studies

The liver, spleen and testes were excised for histopathological analysis, sperm motility and sperm count were also carried out. The organs were preserved in 10% formalin for histological studies so as to detect pathological changes.

Adverse event assessment methods

All the rats were daily inspected visually for possible morphological

Table 1. Sperm analysis for the therapeutic treatment group along with controls.

Group	Sperm count (×10^6/ ml)	Sperm motility (%)
83.3 mg/kg.bd.wt	53.90^a±0.90	79.9 ^{b}±0.95
166.6 mg/kg.bd.wt	48.80 ^{b}±7.00	78.7 ^{b}±1.30
250.0 mg/kg.bd.wt	54.50 ^{a} ±0.70	71.7 ^{c}±0.25
C_p	47.1 ^{b}±3.10	74.2 ^{f}±1.50
C_m	53.9 ^{a}±0.50	63.0 ^{h}±1.25
C_{01}	51.80^{ab}±1.00	90.7 ^{a}±0.70

*Means with different superscript within a column are significantly different at P<0.05. C_p, group administered with *Pseudomonas aeruginosa* only; C_m, group administered with mycomeat only; C_{01}, feed and water only.

Table 2. Sperm analysis for the prophylactic treatment group along with controls.

Group	Sperm count (×10^6/ ml)	Sperm motility (%)
83.3 mg/kg.bd.wt	46.9 ^{a} ±0.90	84.5 ^{d}±1.20
166.6 mg/kg.bd.wt	49.5 ^{ab}±0.70	77.5 ^{e}±3.10
250.0 mg/kg.bd.wt	51.4 ^{ab}±1.00	68.4 ^{g}±1.65
C_p	47.1 ^{a}±3.10	74.2 ^{f}±1.50
C_m	53.9 ^{b}±0.50	63.0 ^{h}±1.25
C_{01}	51.8 ^{ab} ±1.00	90.7 ^{c}±0.70

*Groups with different superscript within a column are significantly different at P<0.05. C_p, Group administered with *P. aeruginosa;* C_m, group administered with mycomeat;C_{01}, feed and water only.

changes; agility, physical appearance and perceived strength.

Statistical analysis

The result for sperm count and sperm motility were expressed as the mean ±SD. Means were analysed using a one-way analysis of variance, followed by the Duncan Multiple Range Test to determine significant differences in all the parameters. Differences with values of P<0.05 were considered statistically significant.

RESULTS

Morphological changes

The rats treated therapeutically with mycomeat appeared very agile, stronger, well-fed and much better than both the prophylactic and untreated but infected control rats. The untreated group appeared haggard, skinny and aggressive. This coupled with the re-isolation of the test organism confirmed that *P. aeruginosa* was able to colonize and initiate infection.

Sperm count and sperm motility

Sperm analysis for the therapeutic treatment group along with controls is as shown in Table 1 while Table 2 shows the sperm analysis for the prophylactic group along with controls. The sperm count in the C_m group was significantly higher (53.9×10^6) than that of the control group (C_{01}), but significantly have the same effect with the group therapeutically treated with 83.3 and 250.0 mg/kg.bd.wt (Table 1). However, the sperm motility was lower than the group given feed and water only; this could possibly result from the pH of the mycomeat.

The group administered with *P. aeruginosa* alone (C_p) had a significantly lower sperm count, and significantly have the same effect with that of Group B_2 (Table 1). Table 2 also shows that the sperm count for A_1 administered with 83.3 mg/kg body weight is not significantly different from that of C_p. The sperm motility for the clean control group (C_{02}) is significantly higher than that obtained for other groups (Tables 1 and 2). Groups B1 and B2 significantly had a lowered sperm motility (Table 1) than that of the clean control group (C_{02}), likewise Groups B3 and C_{01} (Table 1). As shown in Table 3, the therapeutic group appeared better than the prophylactic group.

Histopathological studies

The liver photomicrographs for the prophylactic group showed evidence of vascular congestion in the various concentrations used; however the group prophylactically

Table 3. Pathological conditions associated with the liver, spleen and testis of the experimental groups and controls.

Parameter	Liver		Spleen			Testis		
	Haemorrage	Fatty degeneration	Vascular congestion	Fatty degeneration	Cellular necrosis	Tubular hyperplasia	Mild edema	Celullar degeneration
A_1	+++	-	+++	+++	+	++	-	-
A_2	+	-	+	+++	+	+	-	-
A_3	-	-	++	+++	+	-	+	-
B_1	-	+	-	+++	++	-	-	-
B_2	-	-	-	-	-	-	-	-
B_3	++	++	-	+++	-	+	-	+
C_p	-	++	-	+++	-	++	-	+++
C_m	-	++	-	++	-	++	-	++
C_{01}	-	-	-	+	_	-	-	-

+++, severe; ++, moderate; +, mild; -, absent. C_p, Group administered with *P. aeruginosa;* C_m, group administered with mycomeat;C_{01}, feed and water only. A_1- Group prophylactically administered with 83.3 mg/kg.bd.wt, A_2- Group prophylactically administered with 166.6 mg/kg.bd.wt, A_3- Group prophylactically administered with 250.0 mg/kg.bd.wt, B_1- Group therapeutically administered with 83.3 mg/kg.bd.wt, B_2- Group therapeutically administered with 166.6 mg/kg.bd.wt, B_3- Group therapeutically administered with 250.0 mg/kg.bd.wt.

treated with 166.6 mg/kg.bd.wt of mycomeat (A_2) revealed normal hepatocyte (Plate 1). The spleen for group A_1 showed severe fatty degeneration coupled with cellular necrosis (Plate 2). The sets prophylactically treated with 250.0 mg/kg.bd.wt however revealed a normal seminiferous tubule as shown on Plate 3. The organs of the group therapeutically treated with 166.6 mg/kg.bd.wt are normal showing that mycomeat can preserve the cells of the studied organs at this concentration.

DISCUSSION

The study reveals the potential of mycomeat as a neutraceutical. Mushroom has long been recognized as having several medicinal benefits, however mushroom growing requires great expertise, techniques and investments. The use of mushroom mycelia offers an added advantage over the use of mushroom itself; this include lesser space, shorter production time, less risk of contamination and almost the same medicinal value conferred by the corresponding fruit body. In this study, the medicinal effect of mycomeat against *P. aeruginosa* was established. The histopathological study of the experimental liver revealed normal hepatic cells in both the prophylactic and therapeutic group, as compared to the control group. The mechanisms of the hepato-protective effects are still not clearly defined. However, it is probable that antioxidants, phenolics and radical scavenging activities could be responsible as *L. squarrosulus* is a versatile organism known to secrete a great amount of extracellular polysaccharide into its growth substrate (unpublished data). It has been suggested that antioxidant and radical scavenging activity, modulation of nitric oxide production, maintenance of hepatocellular calcium homeostasis, and immuno-modulatory effects might be involved in hepatoprotection (Wasser, 2005). The therapeutic group galvaged with 166.6 mg/kg.bd.wt were also noted to have normal splenic cells while the prophylactic group showed a severe fatty degeneration, the reason for this is not clear.

Interestingly, the sperm count of the group therapeutically treated showed values that were not dose dependent but significantly higher than the untreated group and the group given feed and water only. However, the sperm motility assay showed a dose-dependent relationship, the higher the dose, the lower the motility of the sperm cells; with all the doses showing a significantly different motility from those of group C_{01}. It is probable that the acidic pH of mycomeat contributed to the reduction in the sperm motility; however, mycomeat clearly supported increased mitotic activity of the sperm cells. This fact is supported by the histopathology study of the testis of group C_m showing an increased number of necrotic sperm cells. An amendment of the pH before administration might lead to fertility improvement, since impairment of spermatogenesis as well as changes like decrease in pH, hypotonic environment, and chemical substances like mucoproteins, alkaline phosphatase and acid phosphatase in spermatogenic cells might lead to the formation of non-viable spermatozoa (Kashinathan et al., 1972).

Valproic acid and carbamazepine has been found to have a similar result on sperm motility. Cohn et al. (1982) used valproic acid and carbamazepine, leading to a

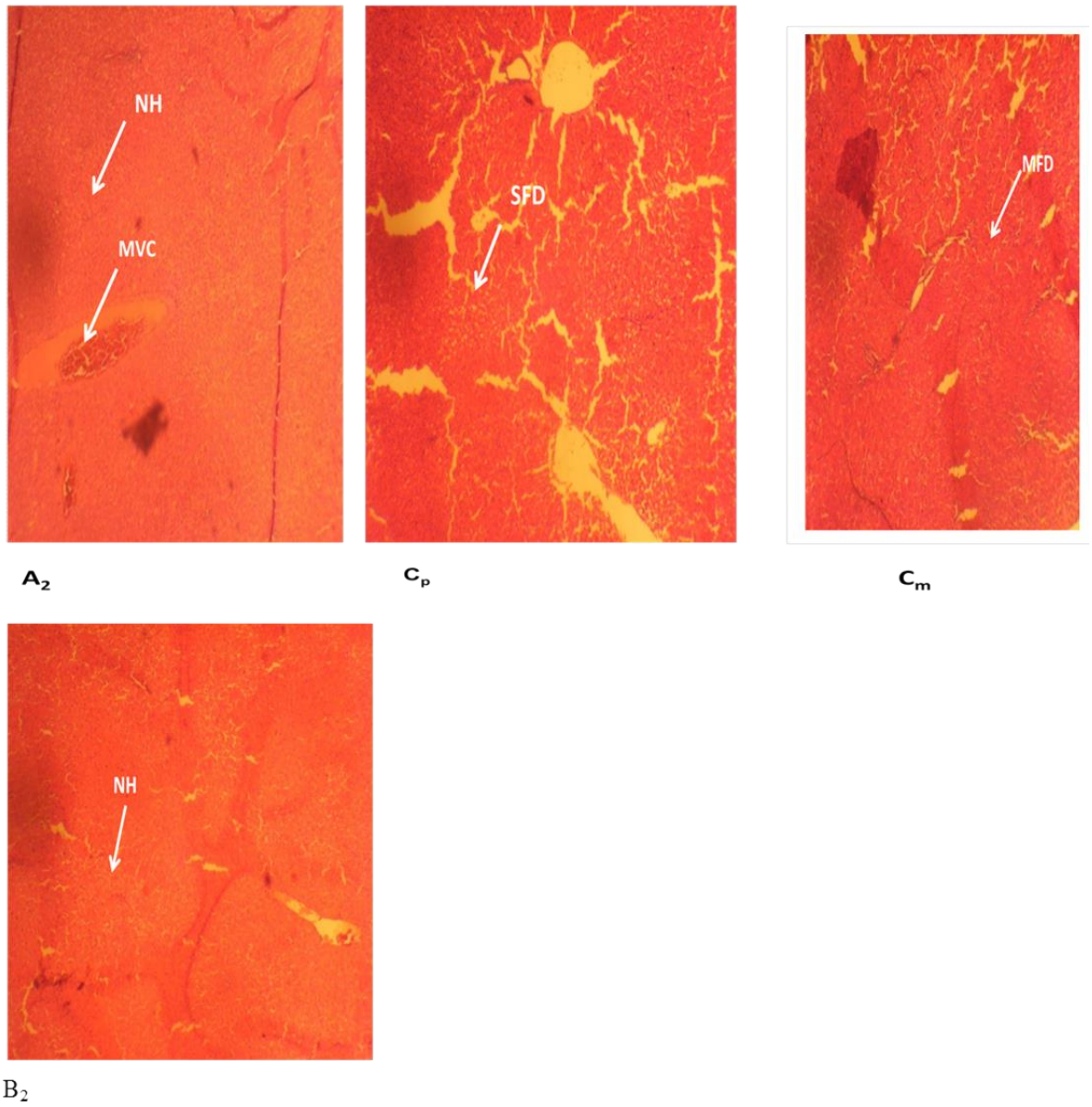

Plate 1. Histological studies of liver of rat in the prophylactic (166.6 mg/kg), therapeutic group (B_2-166.6 mg/kg) along with controls (C_p, Group administered with *P. aeruginosa;* C_m, group administered with mycomeat). MVC, moderate vascular congestion,; MFD, moderate fatty degeneration; SFD, severe fatty degeneration; NH, normal hepatocyte.

reduced sperm content and motility in the experimental rats. On the contrary, Aboua et al. (2012), reported that the intake of red palm oil diet can help protect the male sperm function, leading to improved fertility. A previous research by Chang et al. (2008) found that the supplementation of diet with *Cordyceps militaris* lead to an increased sperm quality and quantity. The report of Khaki et al. (2009) on the spermatogenesis and sperm parameters of rats showed an increase in sperm percentage, motility and viability when ginger was used.

Mycomeat will be applicable in animal husbandry, where meat production is preferred to fertility. However, since there is limited birth control strategy for males, further research on mycomeat can find it useful in countries experiencing overpopulation as a means of birth control. Nevertheless, the effect of mycomeat on the female reproductive system was not covered in this research and could be a future research to be looked into. This study has revealed that *P. aeruginosa* infections could possibly affect some organs, including liver, spleen and testis and could play a part in infertility. The mechanism of action employed by *P. aeruginosa* is still unclear; however *P. aeruginosa* is known to produce hydrogen cyanide (HCN) maximally in the late exponential and early stationary

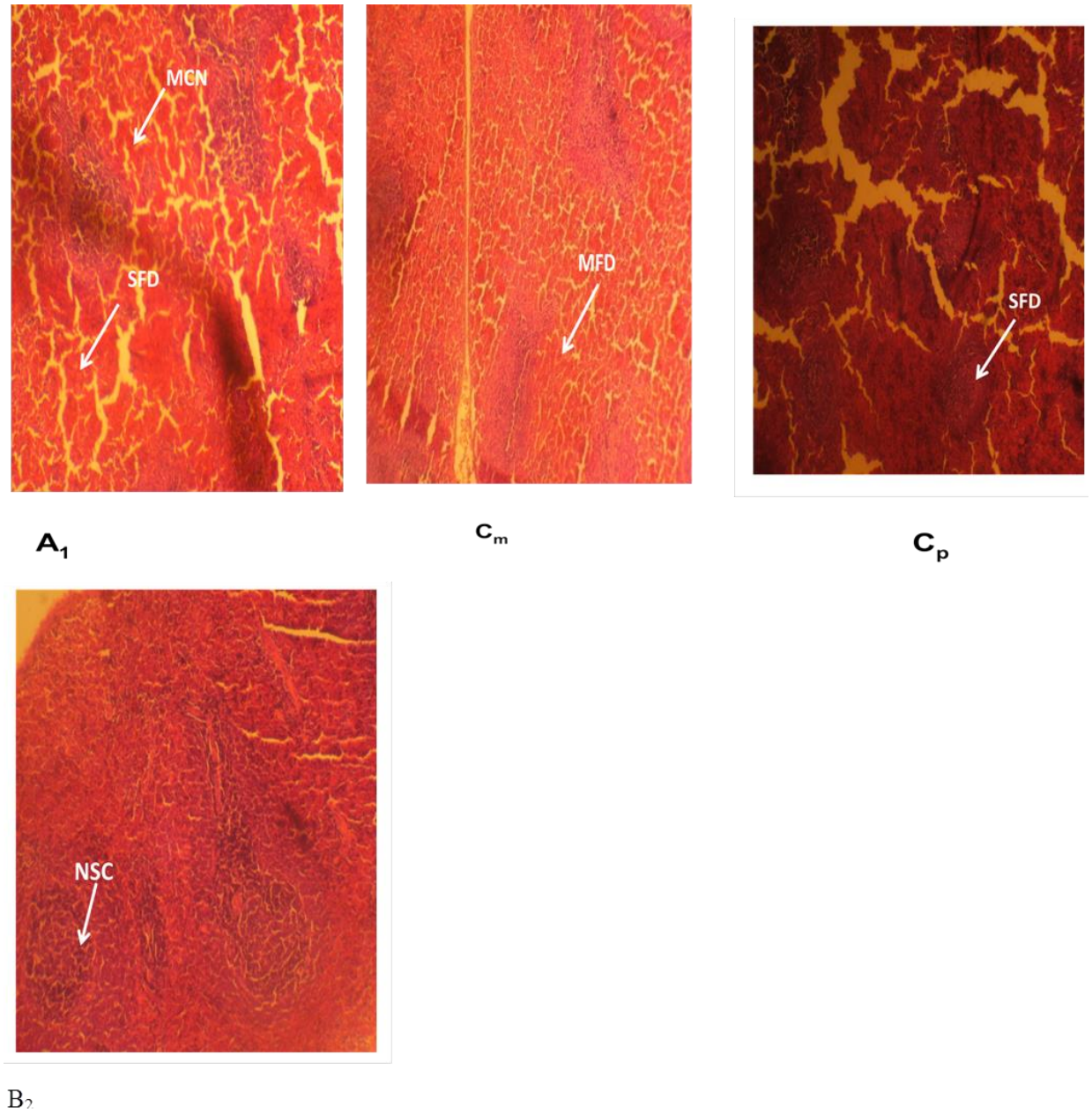

Plate 2. Histological studies of spleen of rats in the prophylactic (83.3 mg/kg - A_1), therapeutic (B_2-166.6 mg/kg) and control groups (C_p, Group administered with *P. aeruginosa;* C_m, group administered with mycomeat). MCN, Mild cellular necrosis; SFD, severe fatty degeneration; MFD, mild fatty degeneration; NSC, normal splenic cells.

phase under microaerophilic conditions (Blumer and Haas, 2000).

Conclusion

This study was able to establish that mycomeat could be useful in preserving body organs, at a dose of 166 mg/kg body weight. It can also be used as a birth control neutracetical for males and in animal husbandry for increasing productivity as induced by castration.

Conflict of Interests

We declare that there is no conflict of interest whatsoever as regards this research work.

ACKNOWLEDGEMENTS

The author appreciates the input of Mr Ayodele S. and Mr Gabriel O. who painstakingly contributed to the success of the research work. Likewise, Mr Owolabi Gbenga for

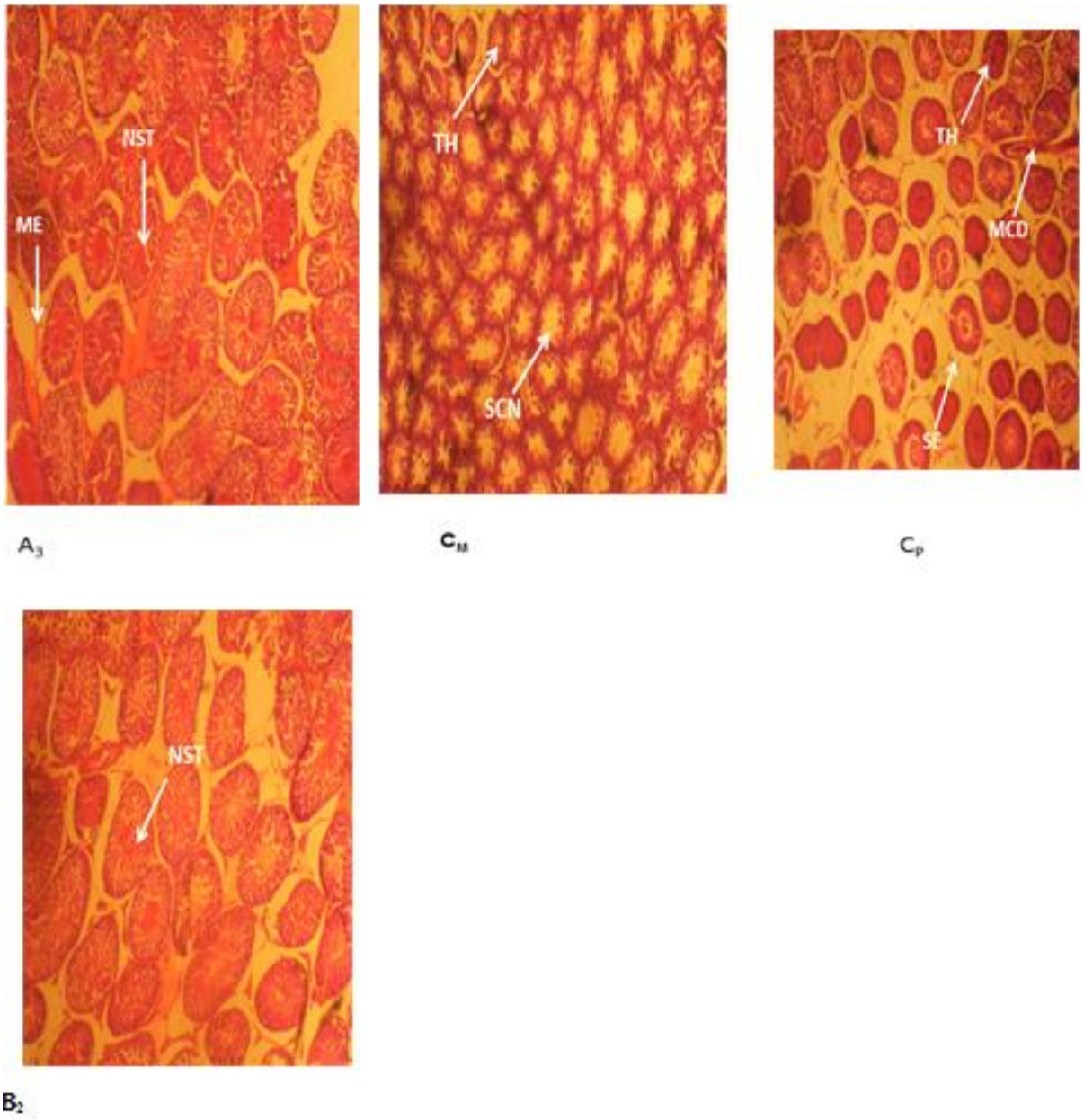

Plate 3. Histological studies of testis of rats in the prophylactic group (A_3-250 mg/kg), therapeutic group (B_2-166.6 mg/kg) along with controls (C_p, Group administered with *P. aeruginosa;* C_m, group administered with mycomeat). ME, Mild edema; NST, normal seminiferous tubule; TH, tubular hyperplasia; MCD, mild cellular degeneration; SCN,s evere cellular necrosis; SE, Severe edema.

the useful input in result interpretation.

REFERENCES

Aboua YG, Brooks N, Mahfouz RZ, Agarwal A, Du Plessis SS (2012). A red palm oil diet can reduce the effects of oxidative stress on rat spermatozoa. Andrologia 44:32-40.

Bamigboye CO, Oloke JK, Adebayo EA, Majolagbe ON, Ayandiran TA, Adegunlola EO (2013). Mycomeat production through the solid state fermentation of soymilk waste by *lentinus subnudus.* J. Nat. Sci. Res. 3:88-93.

Blumer C, Haas D (2000). Mechanism, regulation and ecological role of bacterial cyanide biosynthesis. Arch. Microbiol. 173:170-177.

Chang Y, Jeng KC, Huang KF, Lee YC, Hou CW, Chen KH, Cheng FY, Liao JW, Chen YS (2008). Effect of Cordyceps Militaris Supplementation on Sperm Production, Sperm Motility and Hormones in Sprague-Dawley Rats. Am. J. Chin. Med. 36(5):849-859.

Cohn DF, Homonnai ZT, Gedalia FP (1982). The effect of anticonvulsant drugs on the development of male rats and their fertility. J. Neurol. Neurosurg. Psychiatry 45:844-846.

Collins RA, Ng TB (1997). Polysaccharopeptide from *Coriolus versicolor* has potential for use against Human immunodeficiency virus type I infection. Life Sci. 60:383-387.

Forestier C, Guelon D, Cluytens V, Gillart T, Sirot J, De Champs C (2008). Oral probiotic and prevention of Pseudomonas aeruginosa infections: a randomized, double blind, placebo controlled pilot study in intensive care unit patients. Crit. Care 12(3):R69.

Holder IA (1993). *Pseudomonas aeruginosa* burn infections: pathogenesis and treatment, 275–295. In. Campa M., Bendinelli M., and Friedman H. (ed.), *Pseudomonas aeruginosa* as an opportunistic pathogen. Plenum Press, New York.

Idu M, Osemwegie O, Timothy O, Onyibe HI (2007). A survey of plants used in traditional healthcare by Waja tribe Bauchi State, Nigeria. Plant Arch. 7(2):535-538.

Kashinathan S, Ramakrishnan S, Basu SL (1972). Antifertility effect of *Ocimum Sanctum* L. Indian J. Exp. Biol.10:23-25.

Khaki A, Fathiazad F, Nouri M, Afshin Khaki A, Ozanci CC, Ghafari-Novin M, Hamadeh M (2009). The effects of Ginger on spermatogenesis and sperm parameters of rat. Iranian J. Reprod. Med. 7(1):7-12.

Majolagbe ON, Oloke JK, Adebayo EA, Adewoyin AG, Ayandele A, Bamigboye CO (2013). Study on the Antibacterial Activity of Exopolysaccharides of Lentinus subnudus using swiss albino rats as animal model. Am. Eurasian J. Sci. Res. 8(1):47-52.

Mena KD, Gerba CP (2009). Risk assessment of P. aeruginosa in water. Rev. Environ Contam. Toxicol. 201:71-115.

Oei P (2003). Mushroom cultivation, appropriate technology for mushroom growers (3rd edition), Backhuys Publisher, Leiden, The Netherlands. pp. 46-106.

Oke AJ (2006). Investigation of Immunomodulatory properties of Protein A from a Local strain of *Staphylococcus aureus*, Ph. D thesis Dissertation, Ladoke Akintola University of Technology, Ogbomoso.

Omar MNA, Abdullah N, Kuppusamy UR, Abdulla MA, Sabaratnam V (2011). Nutritional composition, antioxidant activities, and antiulcer potential of *Lentinus squarrosulus* (Mont.) mycelia extract. Evidence Based Complementary Altern. Med. Volume 2011 (2011), Article ID 539356, 8 p.

Osemwegie OO, Eriyaremu EG, Abdulmalik J (2006). A survey of macrofungi in Edo/Delta region of Nigeria, their morphology and uses. Glob. J. Pure Appl. Sci. 12(2):149-157.

Plotnikova JM, Rahme LG, Ausubel FM (2000). Pathogenesis of the human opportunistic pathogen *Pseudomonas aeruginosa* PA14 in *Arabidopsis*. Plant Physiol. 124:1766-1774.

Wasser SP (2005). Reishi or Ling Zhi (Ganoderma lucidum). Enc. Diet. Suppl. pp. 603-623.

academicJournals

Vol. 15(26), pp. 1391-1400, 29 June, 2016
DOI: 10.5897/AJB2016.15248
Article Number: C63C43059225
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Efficacy of chitosan supported organic acaricide extract from *Melia azedarach* leaves on *Rhipicephalus* (*Boophilus*) *microplus* ticks

Priscila Fernanda Pereira Barbosa[1]*, Pablo Peres de Mendonça[1], Rômulo Davi Albuquerque Andrade[2], Ana Carolina Ribeiro Aguiar[1], Andréa Rodrigues Chaves[3] and Fabiano Guimarães Silva[1]

[1]Goiano Federal Institute, Rio Verde Goiás, Brazil.
[2]Federal Institute of Goias, Luizânia,Goiás, Brazil.
[3]Federal University of Goias, Goiânia,Goiás, Brazil.

Received 31 January, 2016; Accepted 10 June, 2016

The *Rhipicephalus (Boophilus) microplus*tick control is mainly performed by chemicals products, but organic acaricides use has higher advantages compared to conventional products. Brazilian cerrado native plants are known for their bioactive potential. Due to this fact, the objective of this study is to evaluate the acaricide action of native cerrado specie, *Meliaazedarach*, known as Santa Barbara in the tick control. Also, the chitosan nanosphere was evaluated on the extract adsorption and release, as a proposal to raise the acaricide phytotherapic profile. The ethanol extract was obtained by cool extraction from dried leaves of the plant. Chitosan nanospheres were obtained by the phase inversion method. Conductometric titration, ultraviolet-visible ("UV-Vis") and Fourier transform infrared spectroscopy (FTIR) analysis were conducted with materials to evaluate the chitosan anchoring ability. *In vitro* test was used in engorged females for each treatment, which consisted of control, raw extract and three treatments with increasing concentrations of 0.2; 0.4 and 1%. The organic product effectiveness of 0.2% concentration was found by observing reduction in the eggs mass compared to control group. The nanomaterial proved capable to anchor and release the acaricide gradually in pH between 6 and 7, which makes it feasible for use in cattle, prolonging the exposure time between the tick and acaricide.

Key words: Cerrado specie, *Rhipicephalus (Boophilus) microplus*tick, ectoparasites, tick control, acaricide, chitosan nanoparticle.

INTRODUCTION

The tick *Rhipicephalus (Boophilus) microplus*is are hematophagous ectoparasites responsible for damage associated with livestock in countries located in tropical and subtropical parts of the world. In Brazil, many cattle

*Corresponding author. E-mail: priscila.barbosa4@hotmail.com.

Figure 1.*Meliaazedarch* tree.

breeds, especially the Europeans, are susceptible to the development of tick parasitic stages, and there are weather changes highly favorable to survival and development of its non-parasitic stages (Junior and Oliveira, 2005). The tick control is mainly performed by chemicals administered by contact or systemically, aiming to combat its parasitic stages. Improper use of these acaricides (individual practices of combat, without applying the recommendations based on appropriate methods of acaricide management) induces resistance strains, growth and strengthening the active principles managed. Thus, perceive the ineffectiveness of some chemical acaricides, and these leave residues in the meat and milkhosts (Broglio-Micheletti et al., 2009).

The need for safer and more effective methods to combat *R. (B.) microplus* has stimulated the search for new acaricides from plant extracts. Thus, it is believed that the plant extracts use isolated or associated to other application methods can lead to slow development of resistance, in addition residues reduction in the meat and milk, because the botanical acaricides are biodegradable (Iannacone and Sludge, 2002). Santa bárbara (*Melia azedarach*) also known as cinamomo is a large tree belonging to the Meliaceae family, represented in Figure 1. The plant insecticidal activity was valued at over 400 insects species, in which over 100 occur in Brazil. This plant insecticidal activity is due to the presence of biologically active compounds, triterpenoids (Brunherotto et al., 2010).

Engorged females immersion tests and larval package made with plant extracts from the leaves, green and mature fruit of this species demonstrated the efficacy in tick control, because there was larvae oviposition inhibition and high mortality rates (Sousa et al., 2008). Thus this plant becomes the subject of many studies about its bioactive potential, since it presents a variability of chemical compounds which can cause death or prevent tick cycle occurrence (Chiffelle et al., 2009). Besides organic acaricide application, the new acaricide profile can be improved by using a technology that promotes the controlled release of the active ingredient gradually. In this context, the biopolymers use for this purpose is an attractive proposition, because it is natural raw materials, degradable and has low cost.

Chitosan is a polymer derived from chitin deacetylation process by enzyme hydrolysis or alkali treatment. It is considered a cationic polysaccharide in neutral or basic pH conditions, contains amino and acetoamino groups, considered a very reactive molecule, which makes it more susceptible to structural changes. Due to the chitosan structure, its use becomes very favorable in compounds binding studies, in addition to controlled release of these in the desired environment (Liu et al., 2008). Considering the biotic potential of the specie *Melia*

azedarach, this study has a goal of verifying the efficacy of ethanol extract from plant leaves in *R. (B.) microplus* control, together with the extract controlled release technology by chitosan nanoparticle surface.

MATERIALS AND METHODS

Nanospheres production

The chitosan solution was prepared with 5% (v/v) acetic acid; the formed mixture was under stirring until complete homogenisation. NaOH solution was prepared at 10% in 100 ml. The solution was standardized until getting a 0.995 correction factor. Chitosan solution was introduced into a "spray drying" system in which the sample passed through a nebulizer to form an aerosol which was gelled in the chitosan nanospheres form. After obtaining the nanospheres, they were washed until it got to pH 7. Subsequently, they were exposed at room temperature to dry (Dias et al., 2008). This method is known as phase inversion.

Plant extracts production

Plant leaves collection

The Santa Bárbara leaves were collected from Goiano Federal Institute (Instituto Federal Goiano) at Rio Verde campus, (17º 48' 16" S, 50º 54' 19" W, 749 m altitude). After collecting them, they were separated from their stems and weighed. After weighing, they were distributed in paper bags and placed in an oven with forced air circulation at a temperature of up to 40°C to dry the leaves. The paper bags weight were checked daily until the weight remained constant. The obtained dried leaves were ground in four knives mill to facilitate the extraction.

Extraction

The extraction process started when the leaves were dried and crushed. They were macerated in 1.5 L of ethanol (Vetec 95%) into an erlenmeyer containing about 300 g of plant mass. The solvent was replaced every 3 days to achieve greater extraction. This process was repeated until the solvent remains translucent. The solvent was volatilized in a rotary evaporator, yielding DCE (dried raw extract).The extract obtained was considered raw extract, but any specific compound was not evaluated by chromatography. It was understood that the *M. azedarch* bioactive potential is due to the presence of triterpenoids.

Anchoring and conductimetric titration

For the raw extract anchoring on the chitosan nanosphere surface, 1 g of nanosphere and 0.5 g of raw extractwas used. 5% (v/v) hydrochloric acid (Sigma Aldrich) solution in 10 ml was prepared. Besides nanospheres and extract, was added 60 mL of distilled water in the solution. The whole system was stirred for 24 h.A conductivity and pH meter was used to check the system conductivity and pH before and during the titration. The titration started at pH 2, and gradually small amounts (uL) of 10% NaOH was added in the system. As pH and conductivity suffered variations, aliquots were removed for each variation in the conductivity for subsequent analysis in the UV-vis and FTIR. All procedures were performed in triplicate.

Fourier transform infrared (FTIR)

Analyses were performed by FTIR-ATR-NIRA- Frontier PerkinElmer simultaneous with the collection of aliquots during titration. Nothing was added in the samples; the analyses just made from the aliquots collected. The relationship between transmittance and wavelength in the spectra was observed. For comparative purposes, aliquots as extract and chitosan solution at 5% were analyzed and observed, alongside the shift of the energy band.

Ultraviolet in the visible region (UV-Vis)

During the conductimetric titrations, the collected aliquots at the points of greatest variation of conductivity were analyzed separately in the UV-Vis Lambda 750 PerkinElmer, which became full scan spectrum (200 - 1000 nm) to obtain the samples gradients absorbance. For this analysis, 200 µL of each sample diluted in 4 ml of ethanol was prepared.

Scanning electron microscopy (SEM)

Realized analysis was done by Scanning Electron Microscope (SEM), JEOL JSM - 6610, equipped with EDS, Thermo scientific NSS Spectral Imaging, using metallizer BALTEC SCD 050 for nanoparticles morphological determination.

In vitro test

For *in vitro* experimentation, *R. (B.) microplus* engorged females in naturally infested cattle on a farm near the Rio Verde city, Goiás were collected. They were washed, dried and separated into 5 groups in the laboratory. The study consisted of 5 treatments, the group I was the control group (ethanol), group II corresponded to the raw extract and the groups III until V were the groups in which the raw extract was diluted in ethanol at concentrations of 0, 2; 0.4 and 1%, respectively. For group III to V, 0.5 grams of chitosan nanosphere treatments were added, and the solution was stirred for 24 hours before starting the tests.

The engorged female ticks were immersed in 20 ml of the solutions corresponding to each treatment for 5 minute, this time being established by Leite (1988). After immersion, the engorged females were dried on paper towels and fixed by adhesive on Petri plates, previously identified (Drummond et al., 1973). 6 engorged females for all groups are performed in triplicate. Subsequently, the specimens were dried on tissue paper and placed in identified Petri dishes in the BOD incubator (28 ± 1°C, 80% humidity) for 14 days. After the laying period, the eggs of each tick female for all treatments were transferred to syringes without tips, sealed with cotton, and again sent to the BOD incubator (28 ± 1°C, 80% humidity) where they were kept for 26 days to evaluate the hatching eggs. Treatments were performed in triplicate and the results were obtained from averages. Data were submitted for analysis of variance and means compared using Tukey test at 5% error probability.

RESULTS AND DISCUSSION

Figures 2 to 4 correspond with chitosan nanoparticles, the *M. azedarch* extract and the extract anchored in the chitosan nanosphere spectra, respectively. It was observed in Figure 1 bands and stretch peaks for the nanosphere chitosan in the region of 3362 cm^{-1} and 1024 cm^{-1}, such regions correspond to angular deformation of the N-H, O-H bond and C-O stretching. In Figure 2 the notaries regions are 3377, 2924, 1696 and 1047 cm^{-1},

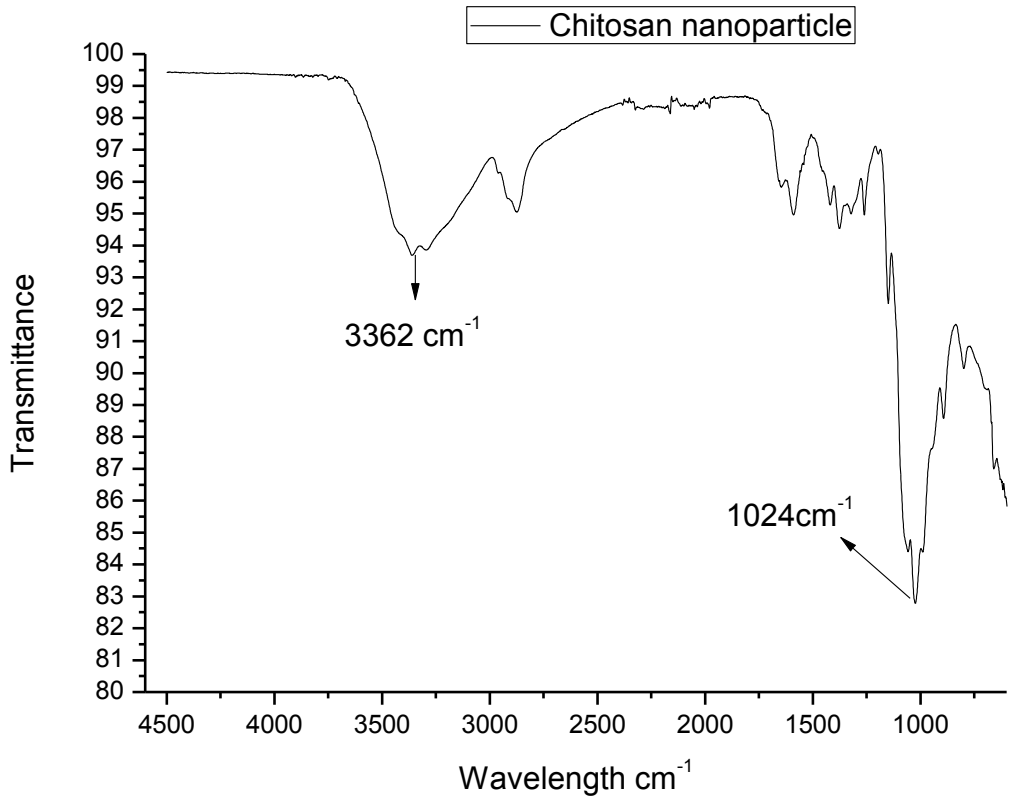

Figure 2. Chitosan nanoparticle spectrum in the FTIR.

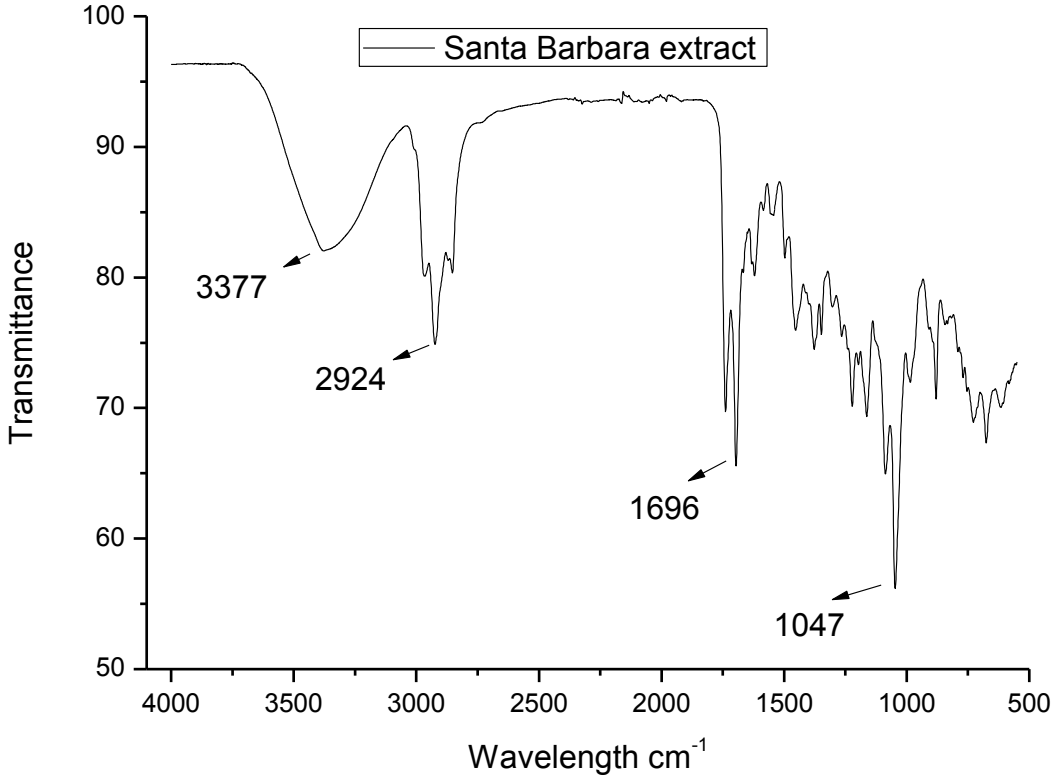

Figure 3. Santa Bárbara spectrum in the FTIR.

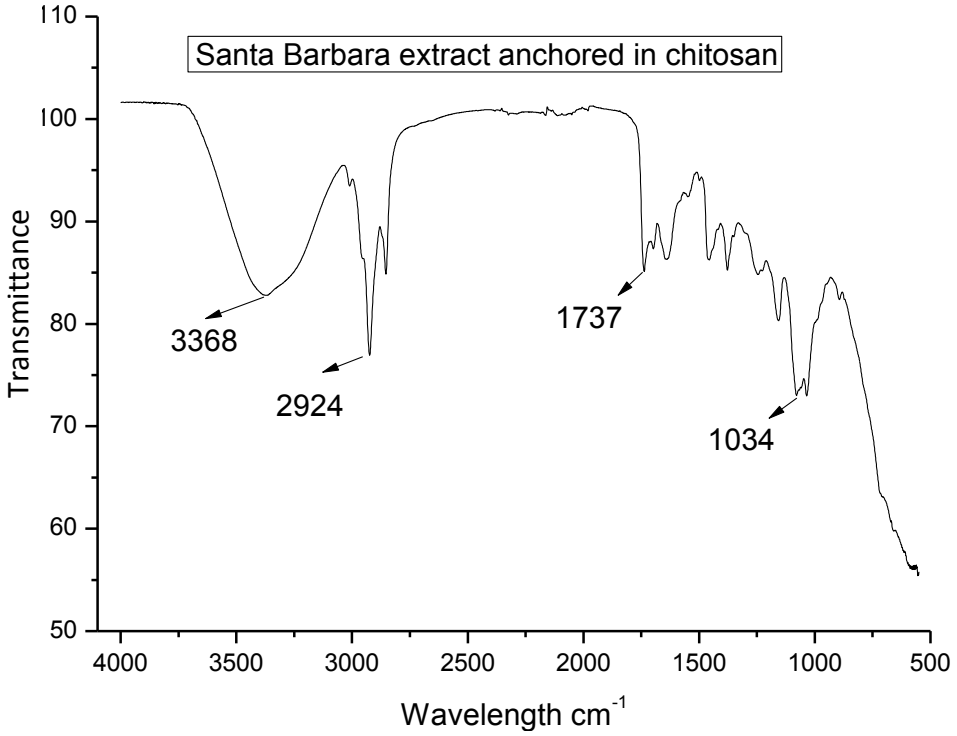

Figure 4. Extract anchored in chitosan spectrum in the FTIR.

wherein the first region is indicated by the presence of O-H bond in alcohols function, ketones and carboxylic acids, the second region corresponds to the C-H bond, the third corresponds to the double stretching of carbon-oxygen and last the stretch C-O of alcohols, esters and others (Aragão and Messaddeq., 2010). All these regions found in *M. azedarch* extract spectra are related to the major metabolite class found in this specie, the triterpenes. The triterpenes are one of the most structurally diverse groups of terpenes, in this class. There are over 40 thousand different structures, with various compounds which serve as important pharmaceutical agents. The triterpenes structure is considerably large, showing some main organic functions, such as alcohol, ester, ether and carboxylic acid (Silva et al., 2014).

When *Meliaazedarch* extract is anchored on chitosan nanoparticle, the spectrum takes another form, raisesnew bands and peaks regarding nanoparticle spectrum without the anchored specie. Figure 4 shows this spectrum, in which the region 3362 cm^{-1} of Figure 2, undergoes displacement emerging band at 3368 cm^{-1} region, and the peak 1024 cm^{-1} also undergoes displacement, showing a peak in the region 1034 cm^{-1}. Furthermore, the other peaks in this figure suffer increase and decrease in the transmittance intensity due to the interactions occurring between the polymer surface and the extract molecules.

During the conductometric titration process aliquots of the reaction medium were collected and analyzed in the FTIR and UV-Vis. It was observed in the FTIR analysis increase and decrease the groups electron density as the pH changed. Figure 5 lists this change in electron density in function of pH change. It was noticed to a greater electron density for spectra were closer to neutral pH, for example at pH 5.95 and 6.43. The peaks near the regions 3500, 1400 and 1000 cm^{-1} had an increase and decrease in transmittance intensity in function of electron density. The denser the peak the greater the amount of adsorbed extract groups in that pH range. According to the results obtained, the pH at which the extract concentration was higher and chitosan molecule was most stable, was close to neutral, between pH 5 and 6.

Baidu and other colleagues (2007) conducted studies on the sorption capacity of metal ions, arsenic III and arsenic V by chitosan surface. One of the analyses carried out by the group involved chitosan FTIR analysis before and after the sorption. The obtained results pointed to differences in the spectra after sorption, in which there was displacement of chitosan characteristic bands and increased in transmittance intensity. The authors have pointed out a possible explanation for this effect, the amount of functional groups present in chitosan after sorption process (Boddu et al., 2007). The extract absorption and release effects from polymer matrix were also evaluated by UV-Vis analysis, by the aliquots collected in the conductometric titration process. The rising of new peaks is shown in Figure 6, beyond the

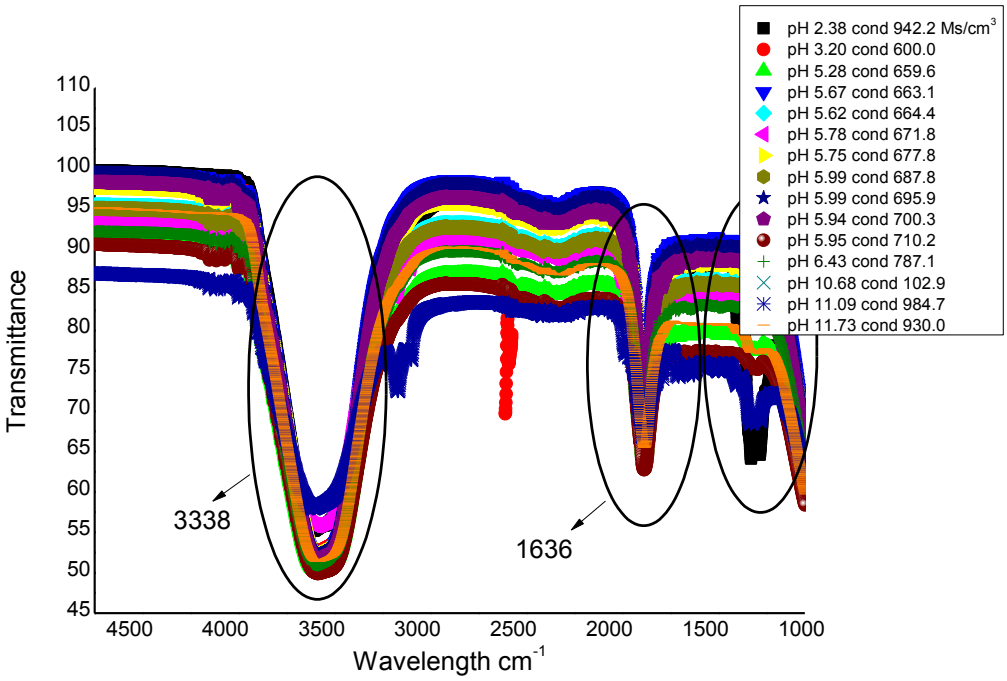

Figure 5. Infrared spectrum of the conductivity variation points obtained during conductimetric titration.

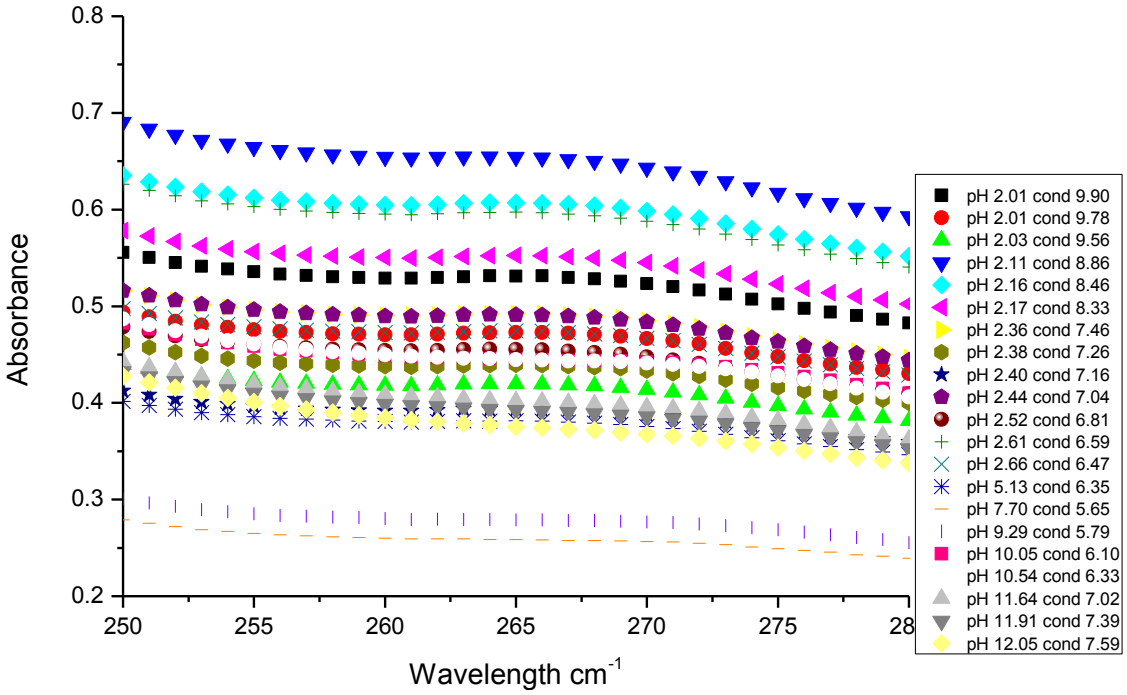

Figure 6. Conductometric titration curves.

increase in absorbance bands according to the pH of the aliquot analyzed.

In the 265 cm^{-1} region, larger absorbance peaks were observed, in which the values found were 0.65 and 0.60 at pH 2.11 and 2.16, respectively. At these points, the chitosan molecules were protonated due to the acid

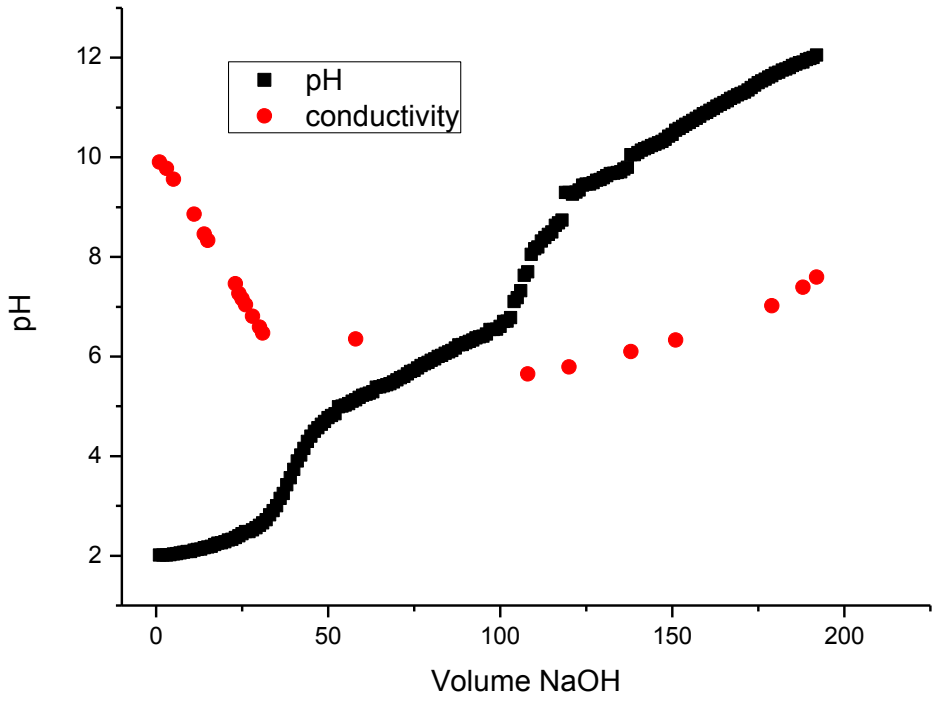

Figure 7. pH and conductivity of the solution during the conductimetric titration.

medium. The amine group is responsible for molecule protonation and deprotonation. Therefore, the pH mentioned might have a higher concentration of adsorbed extract. The conductivity solution change is caused by the absorption and release effects, in this case H^+ ions or extracts molecules. In general, it is noted that the absorbance peaks decrease as the solution becomes more basic, but the last peak is observed at pH 7.40, in the region 265 cm^{-1} with 0.25 absorbance value. In basic medium occurs the amine group deprotonation, releasing if there is adsorbed extract. At this point, the extract concentration in solution is higher because its release for the medium and chitosan nanosphere structure became stable.

Figure 7 corroborates with the results found in UV-Vis analysis. This graph represents the conductivity versus pH from the NaOH volume added in the extract nanosphere chitosan solution. The conductivity decreases and increases with the sodium hydroxide addition, however at a certain point, the pH and the conductivity values cross each other, and from that the conductivity increases. The meeting point is between pH 6 and 7, at this pH can observe greater concentration of extract released by the analysis discussed above. The images taken by SEM show that the methodology used to produce nanoparticles was efficient, displaying uniformity in particle size, about 589 nm, as shown in Figure 8. Mortality was observed in some individuals due to some treatments, but the mortality rate for treatment II (raw extract) is obvious. The bioassay occurred as 100% death in all individuals. But this acaricide use is infeasible due to the amount of plant material used in obtaining them.

In relation to oviposition, the treatments proved to have great potential in reducing the number of egg masses. According to the graph below (Figure 9), the reduction rate ranged between 30 to 100%, and for the treatment II oviposition did not occurred. The effect of increasing concentration was also noted, as the extract concentration increased the eggs mass reduction rate also increased. These results are partially consistent with those obtained by Borges et al. (2003), who used *Meliaazedarach* (Meliaceae) at 0.25% observing *in vitro* complete oviposition inhibition on engorged female immersed in the raw extract of mature fruits extracted with different solvents. Moreover, they observed high larval mortality and high efficacy on engorged females. Although the extract did not kill adult females, it totally or partially inhibited the eggs production. The female mortality in this bioassay was high, especially for the raw extract and this is different from what have been seen.

Statistical test performed with the data obtained can also see from the graph above the similarity between the treatment I and III, corresponding to control and lowest extract concentration groups. This points out that even the lowest concentration used in this study, the *Meliaazedarach* extract, is effective for *R. (B.) microplus* control. Each treatment was performed in triplicate and each bar corresponds one of triplicate for the same treatment. Figure 10 represented by 1, 2 and 3 correspond

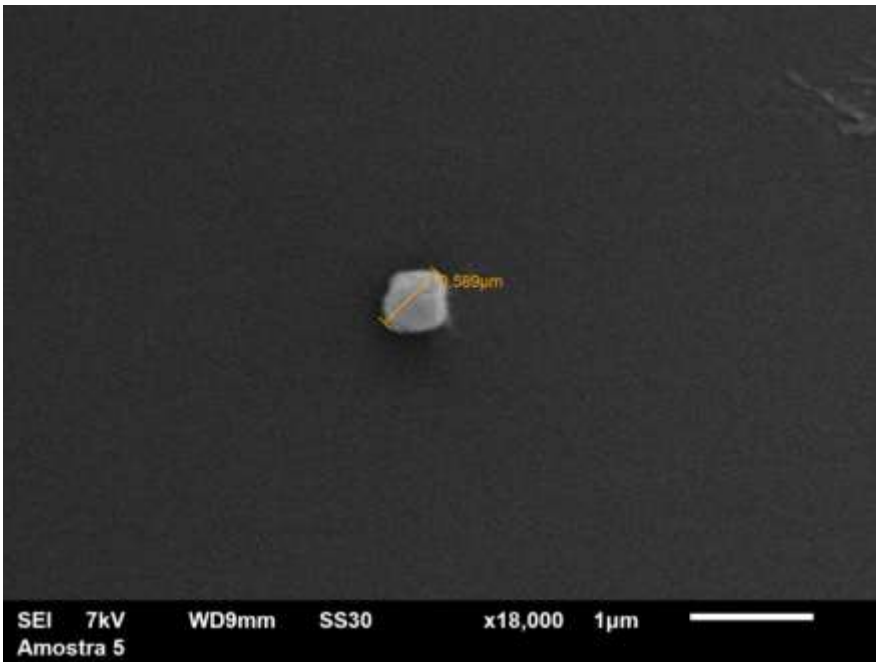

Figure 8. Nanoparticles produced from spray-dry system by gelation method NaOH.

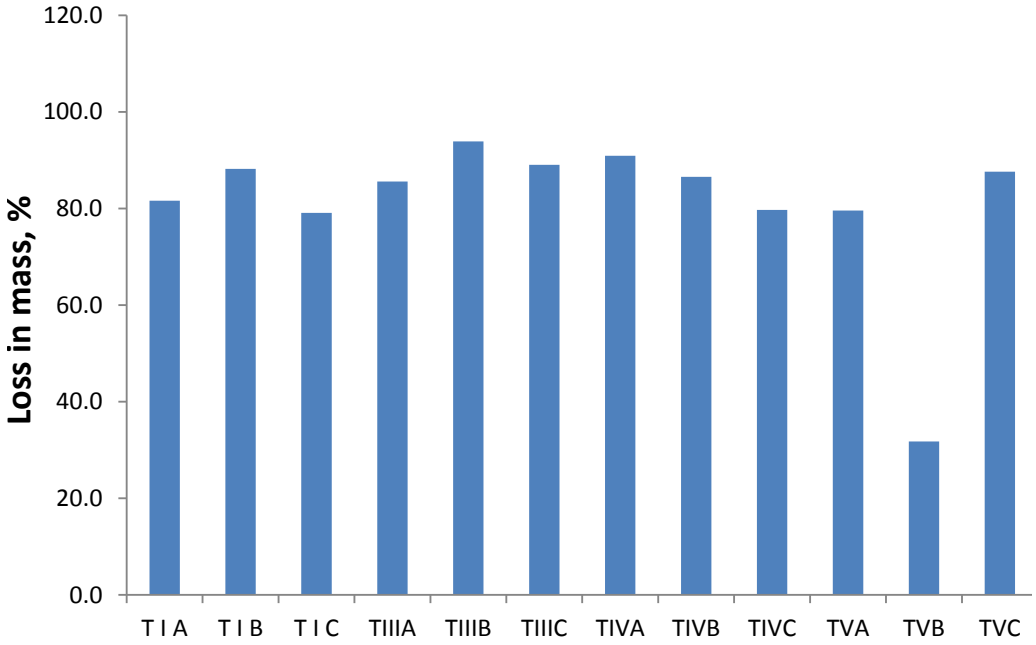

Figure 9. Reduced rate representation in percent of the eggs mass after 14 days from the treatment beginning.

to the control, raw extract and 0.2% concentration, respectively. Clearly, the oviposition for 0.2% concentration treatment decreased considerably compared to the control, and the raw extract did not occur during ovipositon, but all the ticks in this treatment died after7 days of immersion.

Conclusion

The process of production nanoparticles by gelling method in NaOH was effective. It produced a uniform material with 589 nm size. This demonstrates that the spray-drying method is efficient for nanoparticles

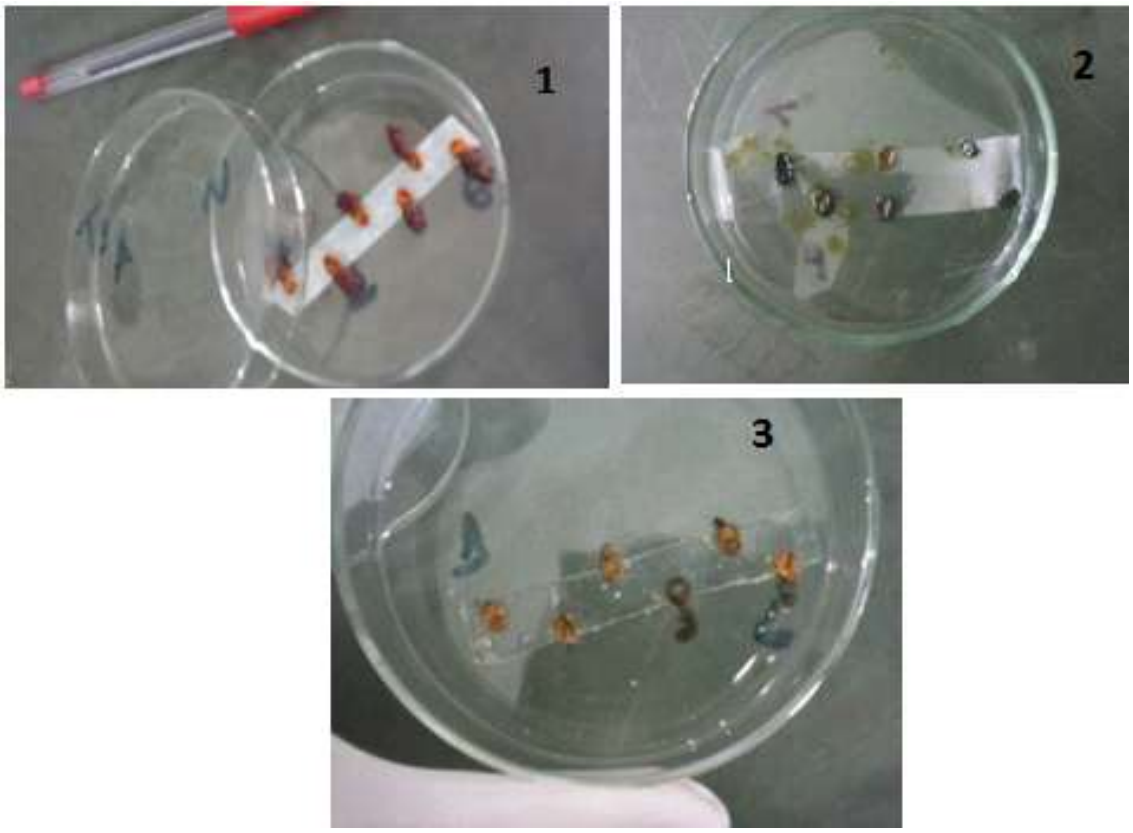

Figure 10. Control treatment, raw extract and concentration of 0.2% oviposition after 14 days from the beginning of the bioassay.

production. The chitosan nanosphere proved to be an effective biopolymer adsorption and release *Melia azedarach* extract. This is because, through the spectra of each one and the adsorbed extract on the nanoparticle surface spectrum, two effects were observed: bands displacement and increase of electron density compared with the spectrum before the adsorption. Furthermore, the UV-Vis analysis confirmed this observation. The acid pH extract remained adsorbed, the pH increased was measured and the extract was released by the polymer matrix. The controlled release is more evident in the pH ranges between 6 and 7, which show the efficiency of the material used to raise the organic acaricide profile. More so, the extract performs the *R. (B.) microplus* control, slowly releases the active ingredient, ensuring a longer exposure time between the tick and the acaricide, and preventing its reproductive cycle. Bioassays performed with the ticks for five treatments showed different effects on control, raw extract and the other treatments with increasing concentrations. For raw extract, 100% mortality occurred in all individuals and for some treatments death was observed too, but this was not so significant. Regarding to the eggs mass reduction, the percentage diversified between 30 to 100%, and in most treatments, the percentage was between 80 to 100%. The treatment of 0.2% was significant at 5% Tukey test, which proves the efficiency of the controlled release technique of the *Meliaazedarach* extract and its acaricidal activity.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are thankful to CNPQ (National Counsel of Technological and Scientific Development) for their financial support. We are also thankful to Goiano Federal Institute for providing laboratories, glassworks and the other necessary materials.

REFERENCES

Aragão BJG, Messaddeq Y (2010). Peak separation by Derivative Spectroscopy Applied to FTIR Analysis of Hydrolized Silica. Journal of the Brazilian chemical Society.19 (8):1582-1594.

Boddu VM, Abburi K, Talbott JL, Smith ED, Haasch R (2007). Removal of arsenic (III) and arsenic (V) from aqueous medium using chitosan-

coated biosorbent. Water Res. 42(3):633-642.
Borges LMF (2003). *In vitro* efficacy of extracts of *Meliaazedarach* against the tick *Boophilus microplus*. Med. Vet. Entomol. 17(2):228-231.
Broglio-Micheletti SMF, Dias NS, Valente ECN, Souza LA, Lopes DOP, Santos JM (2010). Ação de extrato e óleo de nim no controle de *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini, 1887) (Acari: Ixodidae) em laboratório. Braz. J. Vet. Parasitol. 19(1):44-48.
Brunherotto R, Vendramim JD, Oriani MAG (2010). Efeito de Genótipos de Tomateiro e de Extratos Aquosos de Folhas de *Melia azedarach* e de Sementes de *Azadirachta indica* sobre *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). Neotrop. Entomol. 39(5):784-791.
Chiffelle IG, Huerta AF, Lizana DR (2009).Physical and chemical characterization of *Meliaazedarach*L.fruit and leaf for use as botanical insectide. Chil. J. Agric. Res. 69(1):38-45.
Dias FS, Queiroz DC, Nascimento RF, Lima MB (2008). Um sistema simples para preparação de microesferas de quitosana. Quím. Nova 31(1):160-163.
Drummond RO, Ernst SE, Trevino JL, Gladney WJ, Graham OH (1973). *Boophilus annulatus* and *B. microplus* laboratory tests of insecticides. J. Econ. Entomol. 66(1):130-133.
Iannacone J, Lamas G (2002). Efecto de dos extractos botánicos y un insecticida convencional sobre el depredador Chrysoperla externa. Manejo Integrado de Plagas y Agroecología 65:92-101.
Junior DAC, Oliveira PR (2005). Avaliação *in vitro* da eficácia de acaricidas sobre *Boophilus microplus* (Canestrini, 1887) (Acari: Ixodidae) de bovinos no município de Ilhéus, Bahia, Brasil. Ciênc. Rural 35(6):1386-1392.
Leite RC(1988). *Boophilus microplus* (Canestrini, 1887): susceptibilidade, uso atual e retrospectivo de carrapaticida em propriedades da região fisiogeográficas da baixada do Grande-Rio e Rio de Janeiro, uma abordagem epidemiológica.
Liu Z, Jiao Y, Wang Y, Zhou C, Zhang Z (2008). Polysaccharides-based nanoparticles as drug delivery systems. Adv. Drug Deliv. Rev. 7:1650-1662.
Silva FC, Duarte LP, Vieira FSA (2014).Celastráceas: Fontes de Triterpenos Pentacíclicos com Potencial Atividade Biológica. Revista Virtual Química. 6(5):1205-1220.
Sousa LAD, Soares SF, Ferri PH, Borges LMF (2008). Avaliação da eficácia de extratos oleosos de frutos verdes e maduros de cinamomo (*Melia azedarach*) sobre *Rhipicephalus* (*Boophilus*) *microplus* (ACARI: IXODIDAE).Braz. J. Vet. Parasitol. 17(1):36-40.

academicJournals

Vol. 15(26), pp. 1401-1412, 29 June, 2016
DOI: 10.5897/AJB2016.15259
Article Number: 55EE5BD59228
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Optimization of alkaline protease production by *Streptomyces* sp. strain isolated from saltpan environment

Boughachiche Faiza[1,2]*, Rachedi Kounouz[1,2], Duran Robert[3], Lauga Béatrice[3], Karama Solange[3], Bouyoucef Lynda[1], Boulezaz Sarra[1], Boukrouma Meriem[1], Boutaleb Houria[1] and Boulahrouf Abderrahmane[2]

[1]Institute of Nutrition and Food Processing Technologies. Mentouri Brother University Constantine, Algeria.
[2]Laboratory of Microbiological Engineering and Applications. Mentouri Brother University Constantine, Algeria.
[3]Team Environment and Microbiology (EEM), UMR 5254, IPREM. University of Pau and Pays de l'Adour, France.

Received 6 February, 2016; Accepted 10 June, 2016

Proteolytic activity of a *Streptomyces* sp. strain isolated from Ezzemoul saltpans (Algeria) was studied on agar milk at three concentrations. The phenotypic and phylogenetic studies of this strain show that it represents probably new specie. The fermentation is carried out on two different media, prepared at three pH values. The results showed the presence of an alkaline protease with optimal pH and temperature of 8 and 40°C, respectively. The enzyme is stable up to 90°C, having a residual activity of 79% after 90 min. The enzyme production media are optimized according to statistical methods while using two plans of experiences. The first corresponds to the matrixes of Plackett and Burman in N=16 experiences and N-1 factors, twelve are real and three errors. The second is the central composite design of Box and Wilson. The analysis of the results allowed the selection of two factors having a significant effect on the production of the enzyme (fructose and malt extract), then defining theirs optima (7 g/l of fructose and 12 g/l of malt extract).

Key words: Protease, *streptomyces*, identification, fermentation, optimization.

INTRODUCTION

Proteases are among the most important industrial enzymes, accounting for nearly 60% of total industrial enzymes in the market. These enzymes play an important role in biotechnology and are widely used in the tanning industry, in the manufacturing of biological detergents, meat tenderization, peptide synthesis, food industry, pharmaceutical industry and in bioremediation processes (Bhaskar et al., 2007; Jellouli et al., 2009; Deng et al., 2010). Proteases are ubiquitous, they are found, in plants, animals and microorganisms. Microorganisms are the most interesting source of proteases due to their broad biochemical diversity and bioengineering potentiality. Microbial proteases account for approximately 40% of the total worldwide enzyme

*Corresponding author. E-mail: faizasasi@yahoo.fr.

sales (García-Gómez et al., 2009).

Streptomyces spp. (Family: Streptomycetaceae, order Streptomycetales, Class: Actinobacteria) (Whitman et al., 2012) are the most important group of the Actinobacteria with high G+C content of the DNA. Members of the group produce aerial hyphae bearing chains of conidiospores on their tips when growing on agar (Kieser et al., 2000). Several species of the *Streptomyces* are among the most important industrial microorganisms because of their capacity to produce numerous bioactive molecules, particularly antibiotics. *Streptomyces* species are heterotrophic feeders, which can utilize both complex and simple molecules as nutrients. In addition to antibiotics, *Streptomyces* species liberate several extra cellular enzymes (Gupta et al., 1995). In view of potential applications of protease enzyme and economy of production, an attempt was made to study and optimize the protease production from *Streptomyces* spp. The producer strain is isolated from a water sample of Ezzemoul saltpan, located in Ain M'lila (East of Algeria). The present work aims at (i) morphological, physiological, biochemical and molecular studies of the producer strain, (ii) determination of some properties of this protease and also (iii) optimization of media composition, using statistical methods.

MATERIALS AND METHODS

Microorganism

Streptomyces sp. LS strain originally isolated from Ezzemoul saltpan's water (Algeria) was inoculated onto 10, 20 and 30% skimmed milk agar plates (Harrigan and Mc Cance, 1976) and incubated at 30°C for 7 days. Appearance of clearing zone formed by hydrolysis of skimmed milk was used as indication of a protease producer.

Identification of producer strain

Traditional identification

LS strain colonies were characterized morphologically on different media (ISP_2, ISP_3, ISP_4, ISP_5, Starch casein agar, Glucose asparagine agar, Hickey and Tresner gar) following the directions given by the International *Streptomyces* Project (Shirling and Gottlieb, 1966). Cultural characteristics such as growth importance, aerial and substrate mycelium color and diffusible pigment production, were recorded after incubation for 7, 14 and 21 days at 28°C.

Micro morphological observations were carried out with a light microscope using two different methods: slide culture technique (Zaitlin et al., 2003) and inclined coverslips technique (Williams and Cross, 1971; Holt et al., 1994). The physiological and biochemical characteristics were determined according to the methods of Shirling and Gottlieb (1966), Crawford et al. (1993), Chaphalkar and Dey (1996), and Singleton (1999).

Molecular identification

DNA of protease producing strain was extracted using DNA extraction kit «Ultraclean Microbial DNA Isolation (Mo Bio)» and then 16S rRNA genomic regions were analyzed using 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-GGGCGGWGTGTACAAGGC-3') as forward and reverse primers, respectively. Amplification is carried out in « AB Applied Biosystems Veriti 96 well» thermal cycler, using Taq DNA polymerase. Polymerase chain reaction (PCR) program was 95°C/10 min for initial denaturing, 95°C/45 s, 56°C/45 s, 72°C/1 min for 36 cycles and 72°C/ 10 min for final extension.

PCR products were electrophorized on agarose gel with du Tris Borate EDTA in « Embi Tec Runone ^{TM} » electrophoresis cell, under 100 volts. The gel is finally photographed on a UV table «Pharmacia Biotech. Imager Master® VDS» Purified products were subjected to 16S rRNA sequencing by automated Sanger method (Sanger et al., 1977) using 1387R primer, in «GATC Biotech AG, Germany» laboratory. The sequences are finally corrected using the software «Sequencher v. 4.1.4 (Gene Codes)».

Sequences were analyzed by MEGA 5 software (Tamura et al., 2011) and nBLAST tool at NCBI. The 16S rRNA nucleotide sequence of the isolate was aligned with homologous regions from various actinomycetes, and the phylogenetic tree was constructed by neighbor-joining method (Saitou and Nei, 1987). A bootstrap confidence analysis was performed on 1000 replicates to determine the reliability of the distance tree topologies obtained.

Fermentation protocol and enzyme assay

Two fermentation media are used: M1 medium composed of: peptone 5 g/l, meat extract 3 g/l, gelatin 4 g/l, and M2 medium composed of: yeast extract 4 g/l, malt extract 10 g/l, dextrose 4 g/l. Each medium is prepared at three pH values (4.5, 7 and 8.5). Fermentations were carried out in Erlenmeyer flasks (250 ml) containing 50 ml of the fermentation medium. The broth was inoculated with 10^6 spores/ ml and incubated at 30°C for 7 days with shaking at 100 rpm. After fermentation, supernatant was harvested by centrifugation at 11000 g for 20 min at 4°C. The clear supernatant was used as crude enzyme. LS strain protease activity was measured by of Lenoir and Auberger (1977) method modified by Mechakra et al. (1999).

Study of some protease properties

For determination of optimum pH of the enzyme, the reaction mixture buffer was varied over the pH range 2 to 14. Similarly, enzyme production was also monitored at temperature in the range of 20 to 90°C (in increment of 10°C). For the determination of the thermal stability, the enzyme was incubated at different temperature values (60, 70, 80 and 90°C) for 120 min. The samples were submitted to determination of protease activity every 15 min.

Statistical optimization of protease production medium

The protease production is influenced by various production parameters including nutritional and environmental parameters. For this, the media composition is optimized according to statistical methods while using two plans of experiences. The first corresponds to Plackett and Burman design (Plackett and Burman, 1946) for selection of most appropriate medium components. The second is the central composite design of Box and Wilson (1951) to determine the optimal values of components selected in the first plan.

According to the Plackett and Burman design, 15 independent variables (including 3 dummy variables) were organized in 16 combinations. Each variable was examined at a high level (coded as +1) and a low level (coded as -1).The experimental values of the coded levels are shown in the (Table 1). Plackett and Burman

Table 1. Experimental definition for the Plackett and Burman design.

Factors	Low level (-1)*	High level (+1) *
X_1 : glucose	0	3
X_2: starch	0	3
X_3: fructose	0	3
X_4: maltose	0	3
X_5: error	-	-
X_6: yeast extract	0	10
X_7: malt extract	0	10
X_8: peptone	0	10
X_9: casein	0	10
X_{10}:$NaNO_3$	0	10
X_{11}: error	-	-
X_{12}: Na Cl	0	5
X_{13}:$MgSO_4$	0	0.5
X_{14}: K_2HPO_4	0	0.5
X_{15}: error	-	-

* Concentrations in (g/l).

Table 2. Coded and real variables values of central composite design.

Factors	Levels				
	-α	-1	0	+1	+α
X_3 : fructose	0.172	1	3	5	5.828
X_7 : malt extract	5.172	6	8	10	10.828

design is based on the first order polynomial model:

$Y=\beta_0+\sum \beta_i X_i$

Where Y is the response (protease enzyme production), β_o is the models intercept, β_i is the linear coefficient and Xi is the level of the independent variable. All the experiments were carried out in triplicates.

The central composite design was employed in order to find the optimum levels of significant media components. The two selected factors (in Plackett and Burman design) are studied in central composite designs with 11 combinations. Each variable is studied in 5 levels: (-1) lower level (+1) higher level and a central point (0). (-α) and (+α): two levels determined according to α value which is function of the factors number: for 2 factors α = 1.414. The experimental α value is found according to the equation:

(+ /-) α = experimental α value–central level (0) ***/*** step.

The step is the value which separates the central point (0) from the levels (-1) and (+1). The coded and real variables levels are presented in the (Table 2). Analysis of variance allows estimating the significance of results obtained by centered composite design. And if the test is significant, the effect of the factors (X_1, X_2) on the production of the alkaline protease is expressed in form of a quadratic equation:

$Y = b_0+b_1X_1+b_2X_2+b_{11}X_1^2+b_{22}X_2^2+b_{12}X_1X_2$

Coefficients values (bij) are determined by a of multilinear regression programme. Partial derivatives with respect to 0 gives an equation system, the resolution of this equation gives coded optimal levels.

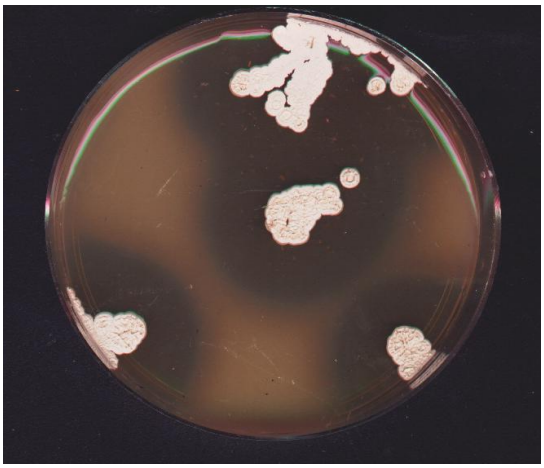

Figure 1. Proteolytic activity shown by LS strain on 20% skimmed milk agar.

RESULTS AND DISCUSSION

Screening for proteolytic activity

LS *Stretomyces* strain was selected for the production of protease on the basis of formation of clear zone near the vicinity of the colony (Figure 1). The dimension of clear zones increases with skimmed milk concentration.

Identification of LS *Streptomyces* strain

Cultural properties of LS strain are presented in the (Table 3). The *Streptomyces* strain grew well on Starch casein agar, ISP_4, ISP_6 and ISP_7. Our isolate produced cream, beige or yellow brown mycelia on all media with brown yellow to yellow diffusible pigment. While the mature spore mass was belonging to yellow series. By studying the micro morphological properties of LS strain, it was found that the aerial mycelia formed unfragmented, branched, straight hyphae bearing non motile cylindrical spores. Spores chains were related to Rectifexibiles (RF) category and contained up to 40 spores per chain.

The physiological and biochemical characteristics of our strain are summarized in the (Table 4). 16S rRNA gene sequence analysis has also been done to elucidate the taxonomic situation and relationship amongst closely related *Streptomyces* spp. LS strain16S rRNA gene sequence was submitted in the GenBank database with accession number KP342331 (2 January, 2015). Comparative analysis of this sequence with the corresponding sequences of other micro organismes obtained from the same database, confirmed the genus assignment and found only 8 most closely related

Table 3. Cultural characteristics of LS strain.

Time	Culture media	Growth characteristics	Pigmentation	
			Substrate mycelium	Aerial mycelium
7th day	ISP_2	Moderate	Brown yellow	Brown yellow(+pigment[1])
	ISP_3	Moderate	Light yellow	Bright cream
	ISP_4	Good	Dark grey	Powdery opaque cream
	ISP_5	Poor	Light yellow	Yellow cream(+pigment[1])
	ISP_6	Good	Beige	Beige
	ISP_7	Good	Light brown yellow	Light brown yellow
	GA	Moderate	Brown yellow	Brown yellow
	SC	Good	Greenish grey	Powdery cream
	HT	Moderate	Light pink	Beige
14th day	ISP_2	Moderate	Brown	Brown (+pigment[1])
	ISP_3	Moderate	Cream	Bright cream
	ISP_4	Good	Greenish grey	Powdery grayish cream
	ISP_5	Poor	Light brown yellow	Brown yellow(+pigment[1])
	ISP6	Good	Beige	Beige
	ISP_7	Good	Brown	Brown (+pigment[1])
	GA	Moderate	Orange yellow	Orange yellow
	SC	Good	Greenish yellow	Powdery cream
	HT	Moderate	Beige	Beige
21th day	**ISP_2**	Moderate	Brown	Brown (+pigment[1])
	ISP_3	Moderate	Cream	Bright cream
	ISP_4	Good	Greenish grey	Powdery grayish cream
	ISP_5	Poor	Light brown yellow	Brown yellow(+pigment[1])
	ISP_6	Good	Beige	Beige
	ISP_7	Good	Brown	Brown (+pigment[1])
	GA	Moderate	Orange yellow	Orange yellow
	SC	Good	Greenish grey	Powdery cream
	HT	Moderate	Beige	Beige

(1) Pigment color change from yellow in acid pH to pink in alkaline pH, GA: Glucose asparagine, SC: Starch casein, HK : Hickey and Tresner.

species with a high degree of relatedness (99%): *Streptomyces flaveus* NBRC 12345, *Streptomyces scabiei* NBRC 12914, *Streptomyces anulatus* NBRC 13369, *Streptomyces olivaceus* NBRC 3152, *Streptomyces tricolor* NBRC 15457, *Streptomyces cavourensis subsp. washingtonensis* NBRC 15391, *Streptomyces praecox* NBRC 13073, *Streptomyces chrysomallus* NBRC 12755.

The Neighbour - Joining phylogenetic tree revealed that the strain formed a monophyletic clade with *Streptomyces tricolor* NBRC15457, with 46% bootstrap support and high similitude degree (99.92%) (Figure 2). The phylogenetic study of Labeda et al. (2012) of almost all described species (615 taxa) within the family Streptomycetaceae, gives à new classification of 130 statistical clades defined at greater than 60% bootstrap support. *Streptomyces tricolor* strain was associated with Clade 116, a well-defined group formed by the strains of *Streptomyces roseodiastaticus*, *Streptomyces bangladeshensis* AAB - 4^T and *Streptomyces rameus* NBRC 15453^T with 99% bootstrap support. All these data suppose that it is probably the same species or closely related species (as *S. bangladeshensis* has different morphological characters of the other three).

Table 5 reports the characteristics of the four strains, it appears clearly that LS strain has different morphological characteristics of other strains with the exception of *S. bangladeshensis* which is the closest to our strain with a yellow color and rectiflexibile aerial mycelium form, however, differences are observed in the assimilation of rhamnose, sucrose, lactose and raffinose, as well as H_2S production, and other physiological characteristics. Therefore, LS strain is different from other species listed in the 116 group including *S. tricolor* (the closest at the phylogenetic tree) and *S. bangladeshensis* (the closest one regarding the phenotypic characters): our

Table 4. Physiological and biochemical characteristics of LS strain.

Test	Results
Degradation of :	
D - glucose	+
L - rhamnose	-
D - mannitol	+
Mannose	+
D - xylose	+
Dextrine	+
D - galactose	+
Sucrose	-
L - arabinose	+
D - fructose	+
Maltose	+
Lactose	+
Raffinose	-
i - inositol	+
Cellulose	+
Production of melanoide pigment on:	
ISP $_6$	- [(1)]
ISP $_7$	+
Hydrolysis of :	
Starch	+
Gelatin	+
Casein	+
Skimmed milk hydrolysis :	
Peptonisation	+
Coagulation	-
Nitrate reduction	+
NaCl tolerance	7 %
Growth at different temperature values :	(dry weight/40 ml)
4°C	-
22°C	+ (22.45 mg)
28°C	+ (50 mg)
37°C	+ (35 mg)
45°C	-
Growth at different pH values :	
5.5	+ [(2)]
6	++
6.5	+++
7	++
7.5	++
8	++
Citrate utilization	-
H_2S production	-
Indole production	-
Tryptophane desaminase test	-
Decarboxylase test:	
Arginine	-
Lysine	+
Ornithine	-
Urease test	+

[(1)]Fot the tests : (+): Strain growth or positive test reaction; (-): No growth or negative test reaction.[(2)] Growth at different pH values: (+++) : Good, (++) : moderate, (+) : poor.

Streptomyces strain is probably new specie. Indeed, the origin of LS strain (isolated from Saltpan) suggests that the species adapted to this extreme environment are eventually particular.

Protease activity assay

After primary screening, secondary screening of protease activity was done by quantitative method, LS strain showed proteolytic activity on two different broth media with better activity on M1 broth at pH 8 (Figure 3), supposing an alkaliphilic nature of the enzyme. However, no growth was observed at acidic pH on both culture media.

Study of enzyme characteristics

The study of LS strain protease characteristics, showed an optimum pH of 8 (Figure 4), an optimum temperature of 40°C (Figure 5) and especially significant thermal stability (Figure 6). In fact, this enzyme keeps 80% of its activity for 15 min at 90°C. After 120 min at the same temperature, the activity decreases to 39%.

Statistical optimization of protease production medium

Selection of the significant factors for protease production using Plackett and Burman design

Protease activities on different media of Plackett and Burman design are shown in Figure 7. Medium 6 has shown the best activity of alkaline protease, this medium is composed of 3 sugars (glucose, fructose and maltose), malt extract and 3 salts (NaCl, $MgSO_4$, K_2HPO_4). The lower proteolytic activity was observed on media 8 and 12. They contain glucose but no fructose or maltose in addition to the presence of $NaNO_3$ and NaCl.

Statistical analysis and modeling results were used to measure the effect of each factor and its significance level on the production of protease by strain LS. The results of this analysis shown in the (Table 6), showed a significant effect of X_3 and X_7 factors at 70% (with Students values of 1.35 and 1.26 respectively). However, a negative effect of X_9 and X_{10} factors is found with negative statistical effects, coefficients and Student values. Y response or protease production can be expressed by the following regression equation:

$$Y=239.5+6.3X_1+22.0X_2+43.2\ X_3+16.2\ X_4+21.8\ X_6+33.2\ X_7+16.3\ X_8-0.1\ X_9-13.8\ X_{10}+26.8\ X_{12}+11.2\ X_{13}+14.3\ X_{14}$$

Factors or culture media components, having a significant effect on alkaline protease production by LS

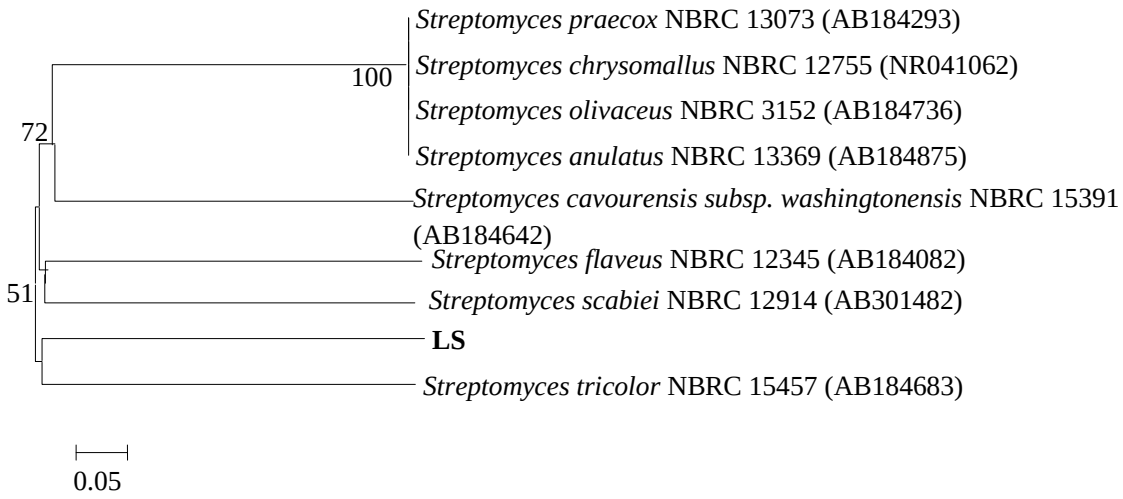

Figure 2. Neighbour-Joining phylogenetic tree based on 16S rRNA gene sequences showing LS strain and the nearest related taxa. (Numbers at nodes indicate percentages of 1000 bootstrap resamplings. Bar equals 0.05 nucleotide substitutions per site).

Table 5. Comparison of phenotypic characteristics of LS strain with related species of the group 11.

Phenotypic characteristics	**LS strain**	***Streptomyces tricolor*** **(Williams et al., 1989)**	***Streptomyces roseodiastaticus*** **(Williams et al., 1989)**	***Streptomyces rameus*** **(Williams et al., 1989)**	***Streptomyces bangladeshensis*** **(Al-Bari et al., 2005)**
Morphology:					
AM color	Cream, yellow to brown yellow	Grey	Grey	Grey	Yellow
SM color	Yellow to greenish grey (change to pink in basic medium)	Yellow, red or blue	nd	nd	Beige
DP	Brown yellow to brown (change to pink in basic medium)	Blue	No MP	No MP	Yellow. No MP
SCT	RF	S	S	S	RF
SNC	40	nd	nd	nd	8 to10
SF	cylindrical	nd	nd	nd	Nd
Hydrolysis of :					
D-glucose	+	nd	+	+	+
L-rhamnose	-	nd	+	-	+
D-mannitol	+	nd	nd	+	+
Mannose	+	nd	nd	nd	+
D-xylose	+	nd	+	+	+/-
Dextrine	+	nd	nd	nd	nd
D-galactose	+	nd	nd	+	+
Sucrose	-	nd	nd	+	+
L-arabinose	+	nd	+	+	+
D-fructose	+	nd	nd	+	+
Maltose	+	nd	nd	nd	+
Lactose	+	nd	nd	nd	-

Table 5. Contd.

Raffinose	-	nd	nd	+	+
i-inositol	+	nd	nd	-	+
Cellulose	+	nd	nd	nd	Nd
Other :					
H_2S production	-	nd	nd	nd	+
Growth temperature	28°C	nd	nd	nd	20 to 50°C
NaCl tolerance	7%	nd	nd	nd	2%

AM: aerial mycelium, SM: substrat mycelium, PD: diffusible pigment, MP: melanoide pigment, SCT: spore chaine type, SNC: spore number per chain, SF: spore form, RF: rectiflexibile, S: spiral, nd: not determined.

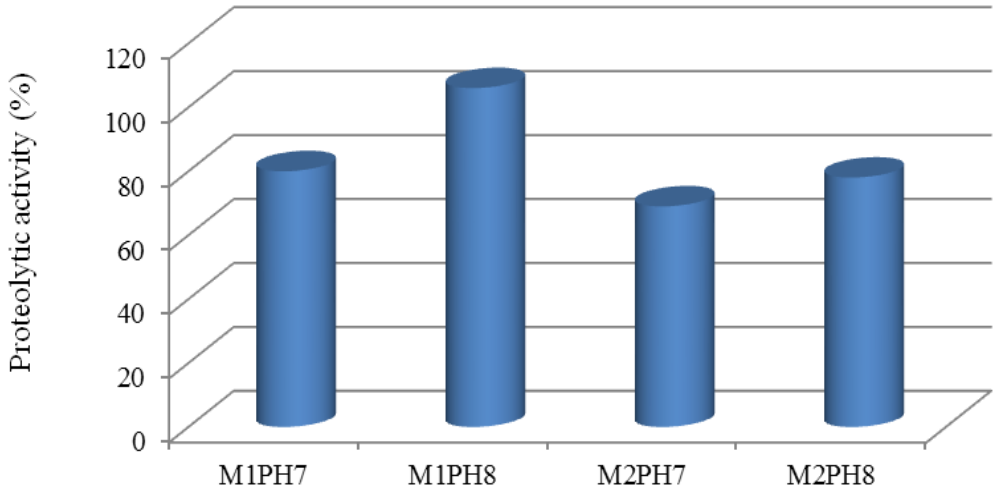

Figure 3. Proteolytic activity on two media broth M1 and M2.

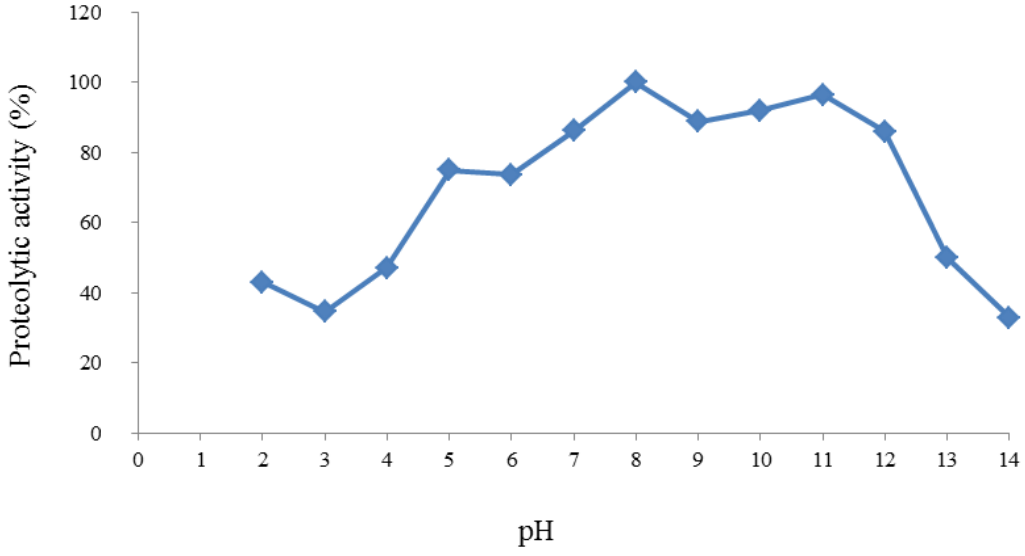

Figure 4. Effect of pH on protease activity.

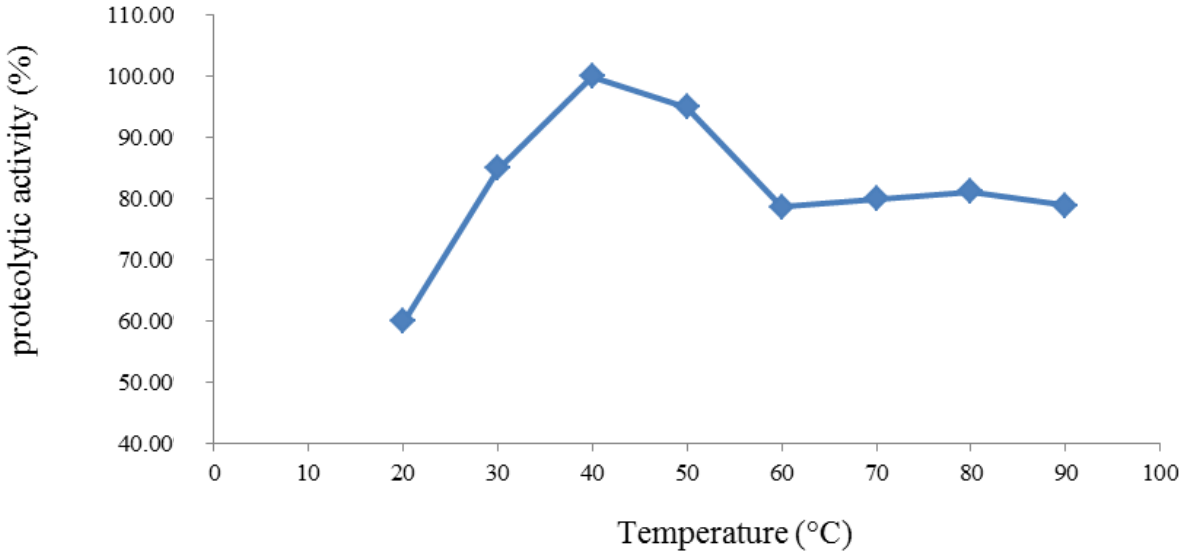

Figure 5. Effect of temperature on protease activity.

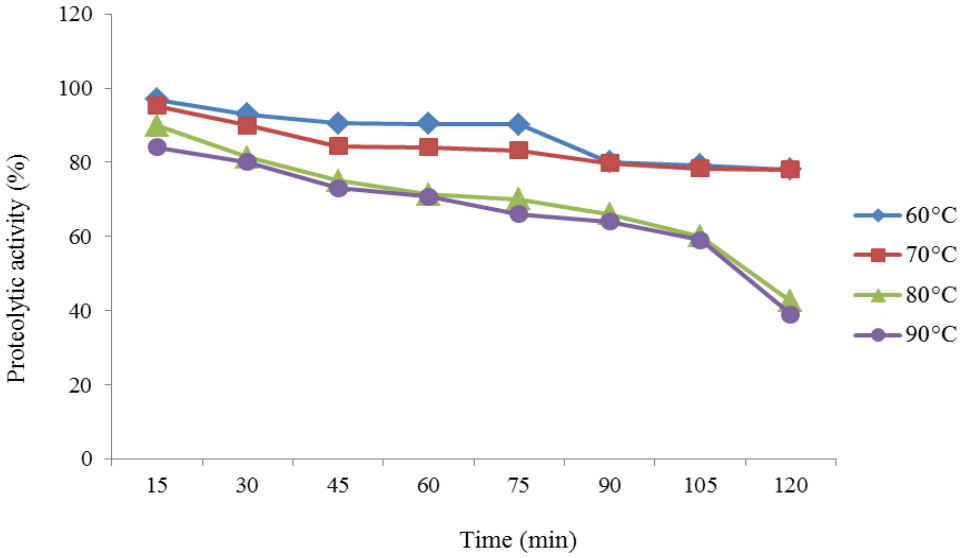

Figure 6. Protease thermal stability study.

strain are:

X_3:fructose.
X_7: malt extract.

(i)Fructose: is a simple sugar found naturally in fruits and honey but can be chemically synthesized. It is used in microbial culture media as a carbon source. Studies have shown that the addition of fructose to the culture medium increases significantly the protease production by *Alternaria* (Mandakini and Shastri, 1983). Others have shown a positive effect of fructose on acid protease production by *Aspergillus* sp. (Radha et al., 2012).
(ii) Malt extract: is obtained after a malted barley flour infusion; it contains, in addition to maltose, amino acids, vitamins and minerals. Several studies have demonstrated its positive effect on alkaline protease production from *Streptomyces* genus (Mostaf et al., 2012). Others found a negative effect of this compound (Elgammal et al., 2012). This is due to the high variability of the genus *Streptomyces*, in fact, their metabolism is different from specie to another, sub specie to another and even from one strain to another. Thus, each strain has its own conditions for growth and primary and

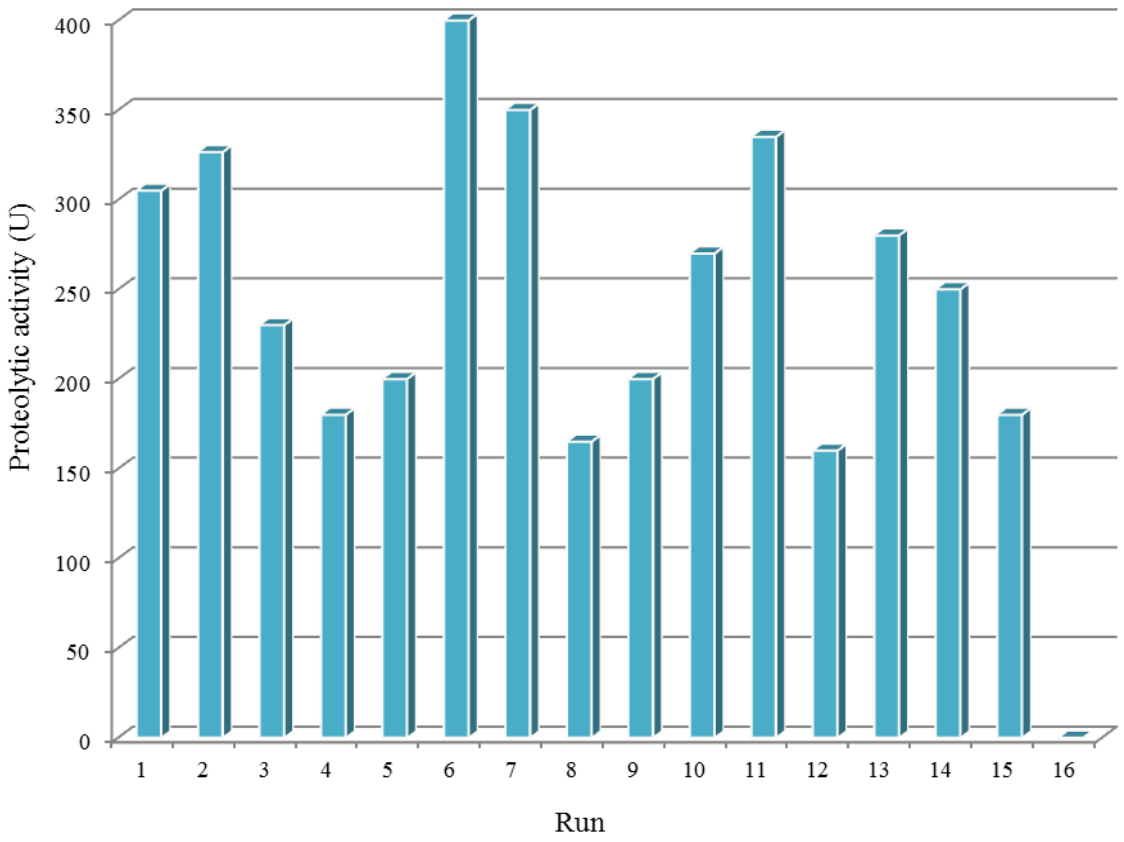

Figure 7. Protease activity according to Plackett and Burman design.

Table 6. Statistical parameters for Plackett and Burman design (Minitab17 software).

Term	Effect	Coefficient	T value	P value
Constante		239.5	7.46	0.005
X_1	12.7	6.3	0.20	0.856
X_2	43.9	22.0	0.68	0.543
X_3*	86.4	43.2	1.35	0.271
X_4	32.3	16.2	0.50	0.649
X_6	43.6	21.8	0.68	0.546
X_7*	66.4	33.2	1.26	0.299
X_8	32.7	16.3	0.51	0.646
X_9	-0.2	-0.1	-0.00	0.998
X_{10}	-27.7	-13.8	-0.43	0.695
X_{12}	53.6	26.8	0.83	0.465
X_{13}	22.3	11.2	0.35	0.751
X_{14}	28.6	14.3	0.45	0.686

T: Student test, p : probability, * : Statistically significant, R^2 = 65 %.

secondary metabolites production. On the other hand, malt extract is an organic nitrogen source which is an inducer of the production of a large amount of protease compared to inorganic sources (Wang et al., 2008).

A negative effect of casein on alkaline protease production by our strain was observed. Indeed, simple substrates such as casein and gelatin have a low solubility and give low enzyme activity yields in liquid medium. Many microbial protease production studies in liquid media have confirmed our results (Joo and Chang, 2005; Laxman et al., 2005; Tari et al., 2006; Chi et al., 2007; Abidi et al., 2008; Hajji et al., 2008 ; Abdelwahed et al., 2014).

In addition, Lazim et al. (2009) showed that the addition

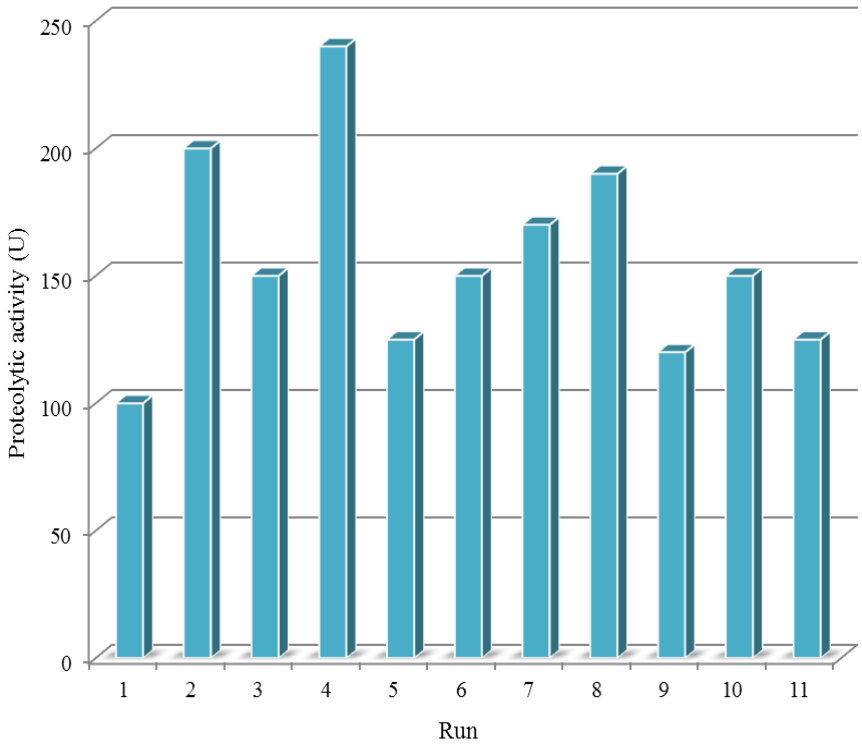

Figure 8. Protease activitiy according to central composite design.

Table 7. Statistical parameters for central composite design (Minitab17 software).

Term	Effect	Coeff	T value	P value
Constant	/	131.7	9.27	0.000
X_3*	63.42	31.71	3.64	0.015
X_7*	36.65	18.32	2.11	0.089
X_3X_3	22.7	11.4	1.10	0.323
X_7X_7*	45.2	22.6	2.18	0.081
X_3X_7	-5.0	-2.5	-0.20	0.847

Coeff : coefficient, T : Student, P : probability, * : Statistically significant.

of casein in the solid culture medium containing wheat bran did not increase the protease production by the *Streptomyces* sp.

Optimization of significant factors for protease production using centered composite design

LS strain proteolytic activities obtained from experimentally Box and Wilson design are shown in the (Figure 8). The best proteolytic activity is obtained on medium 4; it contains both X_3 and X_7 factors at their higher levels, confirming the positive effect of these factors on protease production. The statistical study conducted by Minitab 17 software is presented in Table 7, the Students values (3.64 for X_3 and 2.11 for X_7) confirms the significant effect of both X_3 and X_7 factors observed in Plackett and Burman design. The regression equation coefficients were calculated and the data was fit to a second-order polynomial equation. The response, protease production (Y) by LS *Streptomyces* strain can be expressed in terms of the following regression equation:

$$y = 131.7+31.71\ X_3+18.32\ X_7+11.4\ X_3^2+22.6\ X_7^2-2.5\ X_3X_7$$

The optimal coded values are obtained by calculation of partial derivatives of equation, which are converted into real values giving the optimal concentrations of selected factors (fructose and malt extract) (Table 8). Thus, the medium favoring better proteolytic activity of LS

Table 8. Coded and real values of X_3 and X_7 factors.

Factor	Coded values	Real values
X_3 : fructose	1.99940	7
X_7 : malt extract	1.99940	12

Streptomyces strain is composed of:

(i) Fructose (7 g/l)
(ii) Malt extract (12 g/l)

Conclusions and future perspectives

One of the strategies followed in the search for new metabolites is to isolate the actinomycete bacteria from unexploited ecosystems. Thus, Saltpans which are rare regions in the world seem to be promoter environments to isolate eventually new *Streptomyces* strains which can be sources of interesting or new molecules. The result of LS strain identification confirms this hypothesis. Indeed, the genotypic and phenotypic data show that strain LS forms probably a new specie within the genus *Streptomyces.*

Study of enzyme production and its properties show the high biotechnological potential of this *Streptomyces* strain for the production of the thermostable alkaline protease. Application of statistical methods has allowed the selection of two factors having a significant effect on the production of the enzyme (fructose and malt extract), then theirs optima (7 g/l of fructose and 12 g/l of malt extract). These results open the way to other works like the identification of the LS strain by DNA - DNA hybridization (with related species determined during 16S rRNA gene sequence analysis) and the development of enzyme purification protocol.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Abdelwahed ND, El-Ahmady El-Naggar N, Asem AM (2014). Optimization of alkaline protease production by *Streptomyces Ambofaciens* in free and immobilized form. Am. J. Biochem. Biotechnol. 10:1-13.

Abidi F, Limam F, Marzouki MN (2008). Production of alkaline proteases by *Botrytis cinerea* using economic raw materials: Assay as biodetergent. Proc. Biochem. 43:1202-1208.

Al-Bari MA, Bhuiyan MS, Flores M, Petrosyan P, Varela M, Islam MA (2005). *Streptomyces bangladeshensis* sp. nov., isolated from soil, which produces bis-(2-ethylhexyl) phthalate. Int. J. Syst. Evol. Microbiol. 55:1973-1977.

Bhaskar N, Sudeepa ES, Rashmi HN, Selvi AT. (2007). Partial purification and characterization of protease of *Bacillus proteolyticus* CFR3001 isolated from fish processing waste and its antibacterial activities. Bioresour. Technol. 98:2758-2764.

Box GEP, Wilson KB (1951). On the experimental attainment of optimum conditions. J. Roy. Stat. Soc. B 13:1-45.

Chaphalkar SR, Dey S (1996). Computer assisted identification of *Streptomyces* species with high extracellular protease activity. Actinomycete 7:47-54.

Chi ZC, Wang P, Li HF (2007). Optimization of medium and cultivation conditions for alkaline protease production by the marine yeast *Aureobasidium pullulans*. Bioresour. Technol. 98:534-538.

Crawford DL, Lynch JM, Ousley MA (1993). Isolation and characterisation of actinomycetes antagonists of a fungal root pathogen. Appl. Environ. Microbiol. 59:3899-3905.

Deng A, Wu J, Zhang Y, Zhang G, Wen T. (2010). Purification and characterization of a surfactant-stable high-alkaline protease from *Bacillus* sp. B001. Bioresour. Technol. 101:7100-7116.

Elgammal DE, Elhadedy Moataza Saad M, Moharib SA (2012). Optimization of the environmental conditions for alkaline protease production using *Streptomyces griseus* in submerged fermentation process. Aust. J. Basic Appl. Sci. 6:643-653.

García Gómez MJ, Huerta Ochoa S, Loera Corral O, Prado Barragán LA (2009). Advantages of a proteolytic extract by *Aspergillus oryzae* from fish flour over a commercial proteolytic preparation. Food Chem. 112:604-608.

Gupta R, Saxena RK, Chaturvedi P, Virdi JS (1995). Chitinase production by *Streptomyces viridificans*: its potential in cell wall lysis. J. Appl. Bacteriol. 78:378-383.

Hajji M, Rebai A, Gharsallah N, Nasri M (2008). Optimization of alkaline protease production by *Aspergillus clavatus* ES1 in *Mirabilis jalapa* tuber powder using statistical experimental design. Appl. Microbiol. Biotechnol. 79:915-923.

Harrigan WF, Mc Cance MF (1976). Laboratory Methods in Food and Dairy Microbiology (Academic Press, London). P 464.

Holt JG, Krieg NR, Sneath PHA, Staley J, Williams ST (1994). Bergey's Manual® of Determinative Bacteriology (Williams & Wilkins, USA). pp. 611-703.

Jellouli K, Bougatef A, Manni L, Agrebi R, Siala R, Younes I, Nasri M. (2009). Molecular and biochemical characterization of an extracellular serine-protease from *Vibrio metschnikovii*. Microbiol. Biotechnol. 36:939-948.

Joo HS, Chang CS (2005). Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal: optimization and some properties. Proc. Biochem. 40:1263-1270.

Kieser T, Bibb M J, Buttner M J, Chater K F, Hopwood A (2000). Practical Streptomyces genetics (John Innes Centre, England).pp. 1-43.

Labeda DP, Goodfellow M, Brown R, Ward A C, Lanoot B, Vanncanneyt M, Swings J, Kim SB, Liu Z, Chun J, Tamura T, Oguchi A, Kikuchi T, Kikuchi H, Nishii T, Tsuji K, Yamaguchi Y, Tase A, Takahashi M, Sakane T, Suzuki KI, Hatano K. (2012). Phylogenetic study of the species within the family Streptomycetaceae. Antonie Van Leeuwenhoek 101(1):73-104.

Laxman RS, Sonawane AP, More SV, Rao BS, Rele MV, Jogdand VV, Deshpande VV, Rao MB (2005). Optimization and scale up of production of alkaline protease from *Conidiobolus coronatus*. Proc. Biochem. 40:3152-3158.

Lazim H, Mankai H, Slama N, Barkallah I, Limam F (2009). Production and optimization of thermophilic alkaline protease in solid-state fermentation by *Streptomyces sp*. CN902. J. Ind. Microbiol. Biotechnol. 36:531-537.

Lenoir J, Auberger B (1977). Les caractères du système protéolytique de *Penicillium caseicolum*, II- Caractérisation d'une protéase neutre. Le lait 57:471-489.

Mechakra A, Auberger B, Remeuf F, Lenoir J (1999). Optimisation d'un milieu de culture pour la production d'enzymes protéolytiques acides par *Penicillium camemberti*. Sci. Aliments. 19:663-675.

Mandakini P, Shastri NV (1983). Effect of fructose on extracellular *Alternaria alternata* (Fr.) Keissl. J. Indian Inst. Sci. 64:111-120.

Mostaf A, Moataza MS, Hassan M, Mohsen HS, Helmy MH (2012). Optimization Conditions of Extracellular Proteases Production from a Newly Isolated *Streptomyces pseudogrisiolus* NRC-15. J. Chem. 9:949-961.

Plackett RL, Burman JP (1946). The design of optimum multifactorial experiments. Biometrika 33:305-325.
Radha S, Sridevi A, Himakiran R, Nithya VJ, Prasad NBL, Narasimha G (2012). Medium optimization for Acid protease production from *Aspergillus* sp under solid state fermentation and mathematical modelling of protease activity. J. Microbiol. Biotechnol. Res. 2:6-16.
Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.
Sanger F, Nicklen S, Coulson AR (1977). DNA sequencing with chain-terminating inhibitors. Proc. Nat. Acad. Sci. 74:5463-5467.
Shirling EB, Gottlieb D (1966). Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16:313-340.
Singleton P. (1999). Bacteria in biology, bacteriology and medicine (Wiley, UK). P 236.
Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). MEGA5: molecular evolutionary genetics analysis using likelihood, distance, and parsimony methods. Mol. Biol. Evol. 28:2731-2739.
Tari C, Genckal H, Tokatl F (2006). Optimization of a growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp. L21, Proc. Biochem. 41:659-665.
Wang Q, Hou Y, Xu Z, Miao J, Li G (2008). Optimization of cold-active protease production by the psychrophilic bacterium *Colwellia* sp. NJ341 with response surface methodology. Bioresour. Technol. 99:1926-1931.
Whitman W, Goodfellow M, Kämpfer P, Busse HJ, Trujillo M, Ludwig W, Suzuki KI (2012) Bergey's Manual of Systematic Bacteriology. The Actinobacteria, Part A (Springer Science & Business Media). pp. 1446-1455.
Williams ST, Cross T (1971). Methods in microbiology V4 (Academic Press, London). pp. 295-334.
Williams ST, Goodfellow M, Alderson G (1989). Bergey's Manual of Systematic Bacteriology V4. (Willliams & Wilkins, USA). pp. 2452-2492.
Zaitlin B, Watson S, Dixon J, Steel D (2003). Actinomyetes in Elbow River Basin Alberta Canada. Water Qual. Res. J. Canada. 38:115-125.

academicJournals

Vol. 15(26), pp. 1413-1416, 29 June, 2016
DOI: 10.5897/AJB2016.15340
Article Number: D49F1FD59232
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Recovery of silver from used X-ray film using alkaline protease from *Bacillus subtilis* sub sp. *subtilis*

Amira Hassan Al-Abdalall* and Eida Marshid Al-Khaldi

Department of Biology, Faculty of Science, University of Dammam, El-Dammam, Kingdom of Saudi Arabia.

Received 14 March 2016; Accepted 9 June, 2016

Silver is an important industrial metal used in several areas such as photographic and x-ray films, jewelries, silver wares and electronic objects. Silver is used for photographic film/x-ray film because of its matchless quality as a light-sensitive material for making a photographic image. Silver is not destroyed in the photographic process and it can be reused and recovered. Results have proven that, bacterial alkaline protease can be used to extract silver in 30 min, but its activity decreases with increasing incubation period. Gelatin hydrolysis was monitored by measuring the increase in turbidity of the hydrolysate, which was accompanied by release of protein and hydroxyproline. The protease of the culture filtrate used was 97 U/ml after 30 min, but it decreased to 86.5U/ml after 60 min. After 90 min, it reached 85 U/ml. A great inactivation was recorded after 120 min; it got to 39.5 and 36.5% (U/ml) after 180 min. Gelatin layer was stripped completely within 30 min with 97 U ml^{-1} protease at 50°C and pH 8. At the end of the treatment, gelatin layer was completely removed and the polyester film was left clean. In addition, silver was recovered in the hydrolysate, both of which can be reused.

Key words: Silver recovery, x-ray films, gelatin, alkaline protease, *Bacillus subtilis*.

INTRODUCTION

Alkaline protease is one of the most important enzymes in the commercial field and it occupies a large area in the field of enzyme production. It is widely used in leather industry, diagnosis process, extraction of silver, animal diet production and food processing. For these wide applications, it is now commercially produced (Singhal et al., 2012). Silver is a valuable metal used in photographic and X-ray film, which is considered as an important source of silver metal after recycling of used films compared to other types of films. X-ray films contain about 1.5 to 2% ratio of silver in gelatin-coated film made from polyester layer. And it can restore this quantity of silver by dissolving gelatin layer in alkaline protease to be used for other purposes (Nakiboglu et al., 2003). X-ray film is a rich source of silver, which is distributed in the gelatin layer. Burning is a traditional way of extracting silver. Silver oxidation is followed by electrolysis or chemical treatment of the gelatin layers of X-ray films. All these traditional ways are environmentally unsafe, so enzymatic analysis of the gelatin layer is preferable. For this reason, the considered methods of analysis for enzymatic gelatin are the best alternatives to reduce

*Corresponding author. E-mail: aalabdalall@uod.edu.sa.

Table 1. Silver recovery from waste of X-ray films by alkaline protease.

Time (min)	Optical density ($OD_{660\ nm}$)	Protease activity (U/ml)	Std. error
30	0.2079	97	±10.94
60	0.1781	86.5	±9.88
90	0.1777	85	±9.53
120	0.0861	42.5	±5.12
150	0.0844	39.5	±4.96
180	0.0835	36.5	±4.50

their harmful effects on the environment (Nakiboglu et al., 2003). Gelatin is a protein from animal collagen, which contains a large number of glycine, proline and 4-hydroxyproline residues. Since the emulsion layer on X-ray film contains silver and gelatin, it is possible to break down the gelatin layer using proteases and to release the silver (Nakiboglu et al., 2001). X-ray films are made of polyester which cannot be recycled through traditional methods of silver extraction. Enzyme hydrolysis does not only extract silver from proteins, but also yields the polyester base to be recycled (Gupta et al., 2002). Nakiboglu et al. (2001) and Ahmed et al. (2008) used alkaline protease from *Bacillus subillis, Conidiobolus coronatus* and *Streptomyces avermectinus* to extract silver. Kumaram et al. (2013) found that alkaline protease from *Bacillus* grown on fish ruminants had a high activity in silver extraction. The aim of this work was to detect the use of alkaline protease to extract silver from X-ray films.

MATERIALS AND METHODS

The bacterial isolation

Bacillus subtilis isolated from soil in November 2009 at the Eastern Province of Saudi Arabia was used in this research. Isolation was done in Plant Protection Department, Faculty of Science and Agriculture in King Saud University by Biolog Systems (Al-Yahya et al., 2007). *B. subtilis* sub sp. *subtilis* was the highest isolate from which *Bacillus* alkaline protease was obtained. Identified isolations were evaluated for their ability to produce *Bacillus* alkaline protease (AL-Khaldi, 2014).

Cultural conditions and production of enzyme

The isolate was grown for enzyme production. This was done by incubating it at 37°C for five days in media containing fructose (10 g), potassium nitrate (5 g), NaCl (150 g), dipotassium mono hydrogen phosphate (5 g), magnesium sulfate (0.4 g), $CaCl_2$ (0.2 g) and Tween 80 (10 g) in one liter of sterilized water (AL-Khaldi, 2014). The enzyme was separated by centrifuging at 10,000 rpm.

Alkaline protease used for silver extraction (hydrolysis of gelatin and release of silver)

After washing the X-ray film, it was rubbed over with ethanol. The X - ray film was cut into small pieces (4 × 4 cm), and dried at 40°C for 30 min. Each piece was soaked in a solution containing 500 µl enzyme and 1000.0 µl buffer solution (0.2 M, pH 8). They were incubated at 50°C in a water bath and were shaken (90 rpm) at different periods (30, 60, 90, 120, 150 and 180 min). The turbidity of the reaction mixture (hydrolysate) increased with time and no further increase in turbidity was observed when the hydrolysis was complete. Hence, progress of hydrolysis, that is, turbidity was monitored by measuring the absorbance at 660 nm. Samples were removed at 1 min interval and time required for complete removal of gelatin layer was noted. The resultant color was determined spectrophotometrically at 660 nm (shankal et al., 2010).

Statistical analysis

The statistical analyses were performed in a complete randomized design of three replicates for each treatment. The results were analyzed and compared at 0.05 level of probability using the least significant difference (LSD) and SPSS 16 version of program according to the method of Norusis (1999).

RESULTS

Alkaline protease produced by *B. subtilis* was used in this study. The protease activity of the culture filtrate used was 97 U/ml after 30 min, then it decreased to 86.5% U/ml after 60 min, while after 90 min, it got to 85 U/ml. A great inactivation was recorded after 120 min; it got to 39.5 and 36.5% (U/ml) after 180 min. It was noticed that it took 30 min to decompose the gelatin layer completely at the given experimental conditions (50°C and pH 8). Table 1 and Figures 1 and 2 show the enzyme alkaline protease activity in extracting silver from X-ray films. At the end of the treatment, gelatin layer was completely removed leaving the polyester film clean, and the silver was recovered in the hydrolysate, both of which can be reused.

DISCUSSION

From the results obtained in this study, it was noticed that alkaline enzyme protease was effective in recovering the silver layer during the first 30 min. Subsequently, it went down with increase in the period of incubation. The reason for this decline is due to exposure of the enzyme

Figure 1. X-ray film used in this study as control which contains silver in gelatin-coated film made from polyester layer.

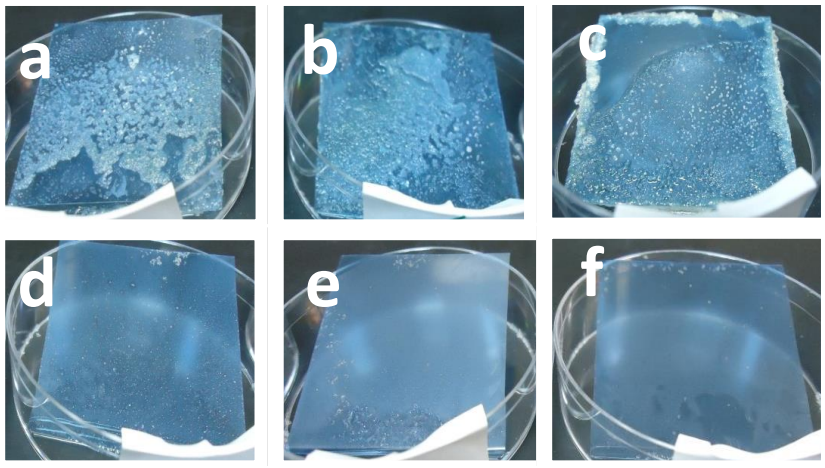

Figure 2. The extract silver layer from treated films by crude alkaline enzyme after: (a) 30 min, (b) 60 min, (c) 90 min, (d) 120 min, (e) 150 min, (f) 180 min, where gradual removal of gelatinous layer was noted.

to a temperature of 50°C, which is the optimum temperature in the different periods of time. This led to breakage of weak peptide bonds, making the enzyme to lose its activity (Bholay et al., 2012). The time factor is important for the stability of the temperature. Alkaline protease proved its activity in extracting silver from used X-ray films. Seid (2011) proved that silver could be extracted after 3 min treatment with alkaline protease at 55°C and pH 10.5, while Shankar et al. (2010) mentioned complete silver extraction after 6 min of using alkaline protease extracted from *C. coronatus*. Nakiboglu et al. (2003) could extract silver in 15 min after using the protease enzyme extracted from *B. subtilis* ATCC 6633; while using enzyme extracted from *Aspergillus versicolor*, Choudhary et al. (2013) extracted silver in 15 min. Also, Foda et al. (2013) extracted silver after 1 h of incubation with alkaline protease.

Silver extraction was also tested at 40°C for 20 min or incubation at 24 h with alkaline protease (Sangeetha et al., 2011). Furthermore, Pathaka and Deshmukh (2012) could extract silver after 24 h. Kumaran et al. (2013) mentioned high activity of the enzyme extracted from *Bacillus* grown on fish remains in silver extraction process.

Conclusion

Recycle of natural mineral resources especially silver metal remains the most practical option to slow down the exhaustion caused by their diminution. This study shows that the alkaline protease of *B. subtilis* sub sp. *subtilis*

has the potential of being reused for extracting silver from used X-ray films in an eco-friendly way.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Ahmed A, Al-Domany A, El-Shayeb A, Radwan R, Saleh A (2008). Optimization, immobilization of extracellular alkaline protease and characterization of its enzymatic properties. Res. J. Agric. Biol. Sci. 4(5):434-446.

Al-Khaldi E (2014). Studies on alkaline protease enzyme produced by a halophilic *Bacillus* isolate from Kingdom of Saudi Arabia. PhD. Thesis in Microbiology. University of Dammam, KSA.

Al-Yahya A, El-Hussieni M, Ibrahim A, Ibrhaim E. A (2007). new biocontrol fungus *trichoderma kongii* in the kingdom of Saudi Arabia isolation and identification. Office J. Saudi Biol. Soc. 14(2):137-144.

Bholay A, More S, Patil V, Niranjan P (2012). Bacterial extracellular alkaline protease and its industrial applications. Int. Res. J. Biol. Sci. 1(7):1-5.

Choudhary V (2013). Recovery of silver from used X-ray films by *Aspergillus versicolor* protease. J. Acad. Indus. Res. 2(1):39-41.

Foda S, Safaa A, Youssef M, Kahil T, Shata M, Roshdy M (2013). Production physiology of alkaline protease by *Bacillus thuringiensis* spp. under solid state fermentation conditions. J. Appl. Sci. Res. 9(3):1975-1984.

Gupta R, Beg K, Lorenz P (2002). Bacterial Alkaline Protease: Molecular approaches and industrial application. Appl. Micro. Biotechnol. 59:15-32.

Kumaran E, Mahalakshmipriya A, Rajan S (2013). Effect of fish waste based Bacillus protease in silver recovery from waste X-ray films. Int. J. Curr. Microb. Appl. Sci. 2(3):49-56.

Nakiboglu N, Toscali D, Nisli G (2003). A novel silver recovery method from waste photographic films with NaOH stripping. Turk J. Chem. 27(1):127-133.

Nakiboglu N, Toscali D, Yasa I (2001). Silver recovery from waste photographic films by an enzymatic method. Turk J. Chem. 25(3):349-353.

Norusis MJ (1990). SPSS/PC + Statistics 6.0 for the IBM PC/XT/AT and PS/2. Library of Congress, USA.

Pathaka A, Deshmukh K (2012). Alkaline protease production, extraction and characterization from alkaliphilic *Bacillus licheniformis* KBDL4: Lonar soda lake isolate. Indian J. Exp. Biol. 50:569-576.

Sangeetha R, Geetha A, Arulpandi I (2011). *Pongamia pinnata* seed cake: A promising and inexpensive substrate for production of protease and lipase from *Bacillus pumilus* SG2 on solid-state fermentation. Indian J. Biochem. Biophys. 48:435-439.

Seid M (2011). Production, characterization, and potential application of a keratinolytic alkaline protease produced by alkaliphilic Vibrio sp. M.Sc. Thesis. Addis Ababa University.

Shankar S, More V, Laxman S (2010). Recovery of silver from waste X-ray film by alkaline protease from *Conidiobolus coronatus*. Kath. Uni. J. Sci. Eng. Technol. 6(1):60-69.

Singhal P, Nigam V, Vidyarthi A (2012). Studies on production, characterization and applications of microbial alkaline proteases. Int. J. Adv. Biotechnol. Res. 3(3):653-669.

academicJournals

Vol. 15(26), pp. 1417-1428, 29 June, 2016
DOI: 10.5897/AJB2015.15129
Article Number: E2BEDC759234
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Flow cytometry approach for studying the interaction between *Bacillus mojavensis* and *Alternaria alternata*

Asma MILET[1]*, Noreddine Kacem CHAOUCHE[1], Laid DEHIMAT[1], Asma Ait KAKI[1], Mounira Kara ALI[1] and Philippe THONART[2]

[1]Laboratory of Mycology, Biotechnology and Antimicrobial Activity, Department of Microbiology, University Mentouri of Constantine, Ain Elbey, Constantine 25017, Algeria.
[2]Wallon Center of Industrial Biology, Agro-Bio Tech, University of Liege Gembloux, 5030, Belgium.

Received 26 November, 2015; Accepted 10 June, 2016

Tomato, *Solanum lycopersicum* is one of the most important vegetable crops consumed in Algeria. Tomato crops are often infected by *Alternaria alternata,* which causes early blight disease. Chemical pesticides are intensively used to protect this plant, which lead to environmental pollution that might endanger animal and human health. The main objective of this study is to select potential biocontrol agents from arid soil as an alternative to chemical products. The phytopathogenic fungus which was isolated from infested tomato leaves, stems and fruits cultured in Constantine-Algeria, was identified as *Alternaria cf. alternata*. Thirty five bacteria isolates were obtained from arid soil in the south of Algeria. Three of the isolates inhibited the growth of *A. alternata*. However, the most potent isolate, E1B3 reached a 75% inhibition rate. The molecular identification of this isolate showed that it was closely related to *Bacillus mojavensis* (KC977492). This strain does not produce chitinase, but does produce lipase, protease and lipopeptides. The interaction between *A. alternata* and *B. mojavensis* was investigated for the first time in this work by flow cytometric analysis. In conclusion, *B. mojavensis* strain was antagonistic to *A. alternata* which could possibly be exploited as a biopesticide in tomato crops management.

Key words: Tomato, *Bacillus mojavensis,* early blight, *Alternaria alternata,* flow cytometry.

INTRODUCTION

Tomato, *Solanum lycopersicum* is among the essential economical and nutritious vegetable crops in the world (Peralta et al., 2005). In Algeria, the land cultivated of tomato represents 12173 Ha which covers 57.36% of the global cultivated areas. It production is ca 10 MQx of which 3.8 MQx is for industrial use (that is equivalent to 95% of the total industrial culture production) (Snoussi, 2009). In spite of the economic importance of tomato, there are major yield losses due to plant diseases. Early blight of tomato is an important and widely distributed

*Corresponding author. E-mail: milet.asmabiotech@gmail.com. Tel: 0780971062.

disease throughout the world. This disease is caused by the fungi *Alternaria solani* and *Alternaria alternata*. *Alternaria* diseases appear usually as leaf spots and blights, but they may also cause damping-off of seedling, stem rots and tuber and fruit rots (Agrios, 1997).

Pesticides have increased crop resistance for over four decades; nonetheless, the emerging, re-emerging and endemic plant pathogens are still challenging crop safety worldwide (Berg, 2009; Gilbert and Haber, 2013). Furthermore, chemicals may leave residues on grains, fruits, vegetables and soil that may be harmful to the ecosystems and human health (Rocha et al., 2014). The development of environmentally friendly crop management practices for combating diseases represents a difficult task. The use of bacterial antagonists such as *Bacillus* (Baysal et al., 2013), *Pseudomonas* and *Streptomyces* (Palazzini et al., 2007; Etcheverry et al., 2009) is a biological alternative solution, commonly called “biocontrol”. This culture management strategy is one of the most rational practices in the integrated management program reducing pesticide use in the environment (Xue et al., 2009).

Bacillus species, including the ubiquitous soil bacterium *Bacillus subtilis*, play an important role in the degradation of soil organic polymers (Emmert and Handelsman, 1999). They produce spores that are resistant to desiccation, heat, UV, irradiation and organic solvents. *Bacillus* spp. are used to manage a wide range of fungal diseases by operating as an antagonist to plant pathogen growth through their production of antibiotics (e.g. iturin, surfactin and fengycin), enzymes that degrade fungal structural polymers (e.g. chitinase and 1,3 glucanase) and production of antifungal volatiles (Fiddaman and Rossall, 1993; Knox et al., 2000; Pinchuk et al., 2002; Leelasuphakul et al., 2006).

Flow cytometry (FCM) is becoming increasingly important in the field of microbial population and community assessment (Müller and Caron, 2010), in medical applications (Walberg et al., 1996; Gauthier et al., 2002), the dairy industry (Rault et al., 2007), alcoholic beverage production (Boyd et al., 2003), and environmental and water system control (Czechowska et al., 2008). It can be combined with cellular markings as carboxyfluorescein diacetate (CFDA, a marker for intracellular esterase activity) (Bunthof et al., 2000; Tanaka et al., 2000; Nguefack et al., 2004; Flint et al., 2006).

CFDA is a lipophilic, non-fluorescent dye, used primarily for the evaluation of cellular enzymatic activity; in fact, it diffuses across the cell membranes, and is converted by unspecific esterases into a membrane-impermeant fluorescent compound, carboxyfluorescein (CF), which is retained in viable cells with intact cytoplasmic membranes (Petit et al., 1993). Single cell analysis in biotechnological applications gives a high-resolution view on a whole cell culture with regards to cellular states of viability, metabolic activity and productivity in addition to cell concentration (biomass) estimation (Hewitt and Caron, 2001).

In this study, an arid region of south Algeria (Sahara) was chosen as a source of new bacterial isolates with potential biotechnological properties. The aim of the present study was to isolate and identify potential biocontrol agents from the Bacillus genus, in order to use them as an alternative for chemical control. The work was carried out using several steps, a) Isolation of *Bacillus* strains antagonistic to *A. alternata*; b) Identification of the most potent isolate, c) Testing for antifungal substances production; d) Study of the effect of *B. mojavensis* strain (E1B3) on *A. alternata* by flow cytometry analysis using the fluorescent dye (CFDA).

MATERIALS AND METHODS

Isolation, identification and pathogenicity test of the pathogenic fungus

Standard tissue isolation technique was followed to obtain the fungal isolates. Three month old tomato plants showing typical early blight symptoms were collected from greenhouses in Hamma Bouziane (Constantine-Algeria). The infected leaves were cut into pieces measuring 2 mm and were surface sterilized in sodium hypochloride solution (0.1%) for 2 min and washed with water. These sections were transferred into Petri dishes containing 15 ml of potato dextrose agar (PDA) and incubated at 27°C for 7 days. Cultures were subcultured onto fresh PDA.

Fungal identification was based on morphological and microscopic tests. For the morphological study of this fungus, PDA medium was used. To confirm the identification, sequencing of the *ITS* region of the nuclear ribosomal operon and a segment of the *Tef I-α* gene was carried out at the Belgian coordinated collections of microorganisms, UC of Louvain (Belgium).

Pathogenicity test of the *Alternaria* isolate were carried out under greenhouse conditions in 2011–2012 year. The inoculum was prepared by culturing the isolate on PDA medium at 27°C for 10 days. Ten milliliters of sterile distilled water was added to each plate and the colonies were scraped with a sterile needle. The resulting conidial suspension was adjusted to 5×10^6 spores/mL and used for the inoculation of tomato plants using an atomizer. After the inoculation, the plants were covered with polyethylene bags for 48 h to promote the entrance of the pathogen and maintain high humidity conditions. After 48 h, bags were removed for the aeration and the entry of light and plants were kept under greenhouse conditions (greenhouse temperature ranged from 26-28°C). Two weeks after inoculation, disease symptoms and severity were recorded.

Isolation of antagonistic bacteria

Bacterial strains were originally obtained from the soil of "Tolga" in Biskra, located in the south of Algeria (Sahara). The isolation was based on the technique which was described by Aneja (2003). One gram of soil sample was suspended in 9 ml of NaCl (0.85%), and decimal dilutions were prepared. 1 mL in ten of each dilution (from 10^{-1} to 10^{-6}) was plated on nutrient agar (NA) and incubated at 28°C for 24 h. Finally, the isolates were stored in inclined agar tubes at 4°C.

Screening bacteria as antagonist of *Alternaria*

The antagonistic effect of bacteria isolated against *Alternaria* sp.

was tested by the dual plate assay. Bacteria were streaked on the PDA and Czapek Dox Agar plate edge (this medium is prepared according to the formula developed by Thom and Church, 1926) and a mycelial plug of *Alternaria* (5 mm) was deposited in the center, approximately 3.5 cm far from the bacteria. Control plates that were not inoculated with bacteria were also prepared. Plates were incubated at 28°C until the fungal growth of control plates reached the edge of the plate. Mycelia growth inhibition was expressed as the percentage of reduction of mycelium expansion when compared with the control plates (without bacteria) (Toure et al., 2004).

Molecular identification of the screened bacteria

The isolate (E1B3) developing the most important antagonist effect against *Alternaria* sp. on PDA medium was screened for further experiments in this work. This bacterium was identified by 16S r-DNA and gyraseA analysis. For that, the total DNA was extracted from bacteria (Potential biocontrol strains) liquid cultures by the wizard Genomic DNA purification kit (Promega), using the manufacturer's instructions. The primers used for the PCR amplification were the universal primers 16S P0 (GAA GAG TTT GAT CCT GGC TCAG) and 16S P6 (CTA CGG CTA CCT TGTTAC GA) for the *DNA-16S* gene, *gyr-A.f* (CAG TCA GGA AAT GCG TAC GTC CTT) and *gyr-A.r* (CAA GGT AAT GCT CCA GGC ATT GCT) for the *gyr-A* gene (Izumi and Aranishi, 2004). The purification of the PCR products was achieved by using the GFX PCR DNA and Gel Band purification Kit. The amplified genes were sequenced using the same primers sited above and the obtained sequences were corrected by the Bio-edit program. The sequences were deposited in Genbank database. To identify bacteria isolates, the DNA sequences were compared with those previously published in Genbank using the BLASTN program.

Cell-wall degrading enzymes

Chitinolytic activity

Chitinolytic activity of the antagonistic strain was evaluated on chitin-agar (CA) plates. The CA agar (per liter) contains the following minerals: K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$ $7H_2O$, $FeSO_4$, $ZnSO_4$, $CaCl_2$ (0.8 g, 0.3 g, 0.3 g, 0.5 g, 0.1 g, 0.001g and 0.1 g, respectively), in addition, it contains; 3 g colloid chitin, 8 g yeast extract, 20 g agar, with pH 6.5. The CA plates inoculated with the bacteria isolates were incubated at 28°C for 5 days. After that, the width (mm) of every clear halo around the antagonistic isolate was measured and recorded as indicator of chitinolytic activity (Nihorimbere et al., 2013).

Protease activity

The protease production was tested by using the casein skim milk as a carbon source (Larpent and Larpent, 1985). The culture medium is composed of 9 g/L casein peptone, 9 g/L yeast extract, 9 g/L of skim milk and 14 g/L agar. An overnight colony was deposited on the agar plate centre, and then incubated at 28°C for one week. Protease activity was highlighted by the presence of a transparent halo around colonies (Nihorimbere et al., 2013).

Lipase activity

The ability of isolate to produce the lipase enzyme was determined by testing the tributyrin hydrolysis. This test was performed by the method described by Larpent and Larpent (1985) with some modifications. An overnight colony was deposited on the centre of the agar plate. After one week of incubation at 28°C, the lipase activity was confirmed by the formation of a transparent halo around the colonies. The lipase production was measured as the ratio of diameters of clearing zone to that of the colony (Nihorimbere et al., 2013).

Lipopeptides production

The lipopeptides (LPs) were analyzed by mass spectrometry. The *Bacillus* strain**s** were grown in agitated flasks (180 rpm) containing the optimum medium at 30°C for 72 h. Cultures were centrifuged at 15000 rpm for 20 min. The supernatant samples were loaded on C18 solid-phase extraction cartridges (900 mg, Alltech) and lipopeptides were desorbed with 100% acetonitrile ACN. The resulting samples were analyzed by reverse phase HPLC coupled with single quad mass spectrometer (HPLC Waters Alliance 2695/diode array detector, coupled with Waters SQD mass analyzer) on a X-terra MS (Waters) 150 9 2.1 mm, 3.5 lm column as previously described by Nihorimbere et al. (2013). In this work, a single elution gradient allowing the simultaneous measurement of all three lipopeptides families was used. Water acidified with 0.1% formic acid (A) and acetonitril (ACN) acidified with 0.1% formic acid (B) were used as a mobile phase. The flow rate was maintained at 0.5 ml/min and the column temperature at 40°C, with a gradient of 35 min (43–80%, vol/vol ACN in 18 min; 100%, vol/vol ACN for 9 min, and 43%, vol/vol ACN in 8 min). Compounds were identified on the basis of their retention times ad compared to the purified standards. The identity of each homologue was confirmed on the basis of the masses detected in the SQD by setting electrospray ionization conditions in the MS as source temperature, 130°C; desolvation temperature, 250°C; nitrogen flow, 500 l/h; cone voltage, 70 V. The positive ion mode was used for analyzing the three families.

Effect of *Bacillus mojavensis* on *Alternaria alternata*

Microbial suspension preparation

A. alternata was cultured on PDA plates, from which, the colony surface was gently scraped off and transferred to a tube containing 50 mL of sterile distilled water. Spores were counted with a hemocytometer, and the concentration was adjusted to 10^6 spores/mL. On the other hand, *B. mojavensis* was cultured in Erlenmeyer flasks containing 250 mL of optimum liquid medium as described by Jacques et al. (1999) and incubated at 30°C on a rotary shaker (100 rpm) for 72 h. The cultures were centrifuged at 12 000 rpm for 30 min at 4°C.

Flow cytometry analysis

In the FCM assay, microbial suspensions of *A. alternata* and *B. mojavensis* were used at 10^6 spores/mL and 10^7 cell/mL, respectively. About 50 µL of *B. mojavensis* biomass was centrally inoculated on PDA medium using a micropipette. After inoculum adsorption, 50 µL of *A. alternata* was centrally inoculated on each PDA medium Petri plate. The cytometry experiments were conducted with three replicates after 3, 5 and 7 days of incubation at 30°C (Rocha et al., 2014 method modified). After the necessary incubation time, cultures were removed from the agar by scraping, transferred into 1 mL Eppendorf containing sterile distilled water and Tween 80 (2 drops/100 mL water), and centrifuged at 10000 rpm for 15 min. The cells were rinsed with the Tween 80/water solution twice and resuspended in 1 mL of phosphate-buffered saline (PBS; 136 mM NaCl, 1.4 mM KH_2PO_4, 8.09 mM Na_2HPO_4,

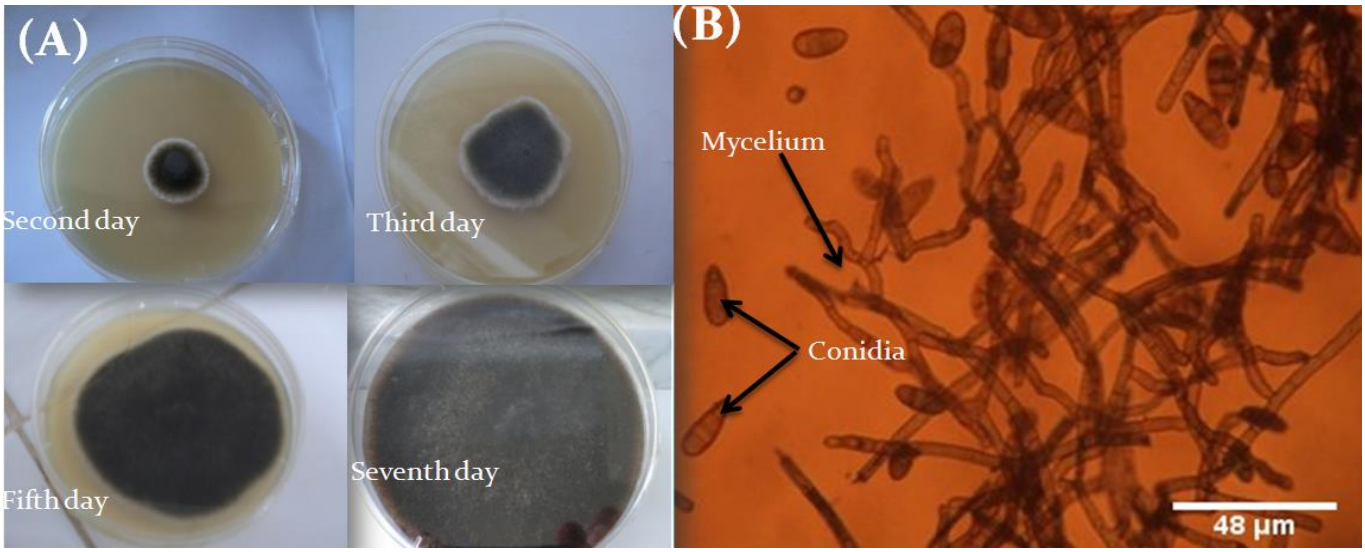

Figure 1. Morphology of *Alternaria* sp. isolated from diseased tomato plants leaves, (A) aspect of macroscopic observation from the third day to seventh, (B) observation of mycelium and conidia under optic microscope (GX40).

12 H_2O and 2.6 mM KCl, pH 7.2). The cell solution was stained with 10 µL of CFDA (a working solution of 100 µg. mL^{-1} was prepared in (PBS) and stored at 4°C in the dark). The mixture was incubated in the dark for 15 min before FCM analysis. The samples were centrifuged at 10000 rpm for 15 min and resuspended in 1 mL of sterile PBS. After a second centrifugation, the final pellet was resuspended in 1 mL of sterile PBS and stored in ice until the analysis. To analyze the influence of thermal treatments (control), 10^6 spores/mL of the *A. alternata* were heated at 90°C for 12 min in a water bath. Cell viability of the heat treated solution was analyzed by FCM after staining with CFDA. One milliliter of a 10 ^{6} spores/mL of *A. alternata* solution was analyzed before and after heat treatment to confirm that dead cells cannot absorb CFDA and also to distinguish between the death and live cells GFI. The cells were incubated with CFDA in the dark for 15 min and stored in ice until the analysis. The same manipulations were carried on bacteria to estimate the effect of heat treatment on cells viability.

The samples were analyzed on a FACScan flow cytometer with an argon laser operating at 488 nm. Green fluorescence of cells stained with cFDA was collected in the FL1 channel (525 ± 20 nm). 40,000 events were collected per sample. The experiment was repeated twice.

Fluorescence microscopy

The same microbial cultures on PDA plates prepared for flow cytometry test as described before, were used for studying the effect of E1B3 on *A. alternata* growth at intracellular and intercellular levels, by fluorescence microscopy using a Zeiss, AXIOSCOP 2MOT.

RESULTS AND DISCUSSION

Isolation, identification and pathogenicity test of pathogenic fungus

One fungus was isolated and purified on PDA plates. This isolate had green black colonies with a very thin white margin and cottony texture (Figure 1A). Conidiophores appear under optic microscope straight, with a brown color and bearing light brown conidia with a short beak at the tip.

The comparison of the fungus, *ITS* and *Tef I-α* genes sequences with those previously published in Genbank showed that this isolate was *A. cf. alternata.* The results of pathogenicity test indicated that *A. alternata* strain was able to infect tomato plants causing typical early blight symptoms (Figure 2).

Isolation, identification and screening bacteria as antagonism of Alternaria

Strains of *Bacillus* spp. are among the most commonly reported biofertilizer and biopesticide bacteria. The characterization of this genus for their antifungal effect has been carried widely on *Bacillus* strains isolated from the rhizosphere of agricultural crops (Beneduzi et al., 2008; Pamela et al., 2010). However, this study is, from the authors' knowledge, one of the few studies exploring *Bacillus* strains isolated from arid soil.

Thirty five bacteria were isolated from arid soil in the south of Algeria (Sahara). Three isolates inhibited the growth of *A. alternata*. However, the most potent strain was the coded bacteria (E1B3) which reached 75% inhibition rate (Figure 3). The antagonism test was performed on PDA and Czapek Dox Agar plates which is a semi synthetic medium, containing sucrose as the sole source of carbon while sodium nitrate as the sole source of nitrogen. Dipotassium phosphate buffers the medium; Magnesium sulphate, potassium chloride, ferrous

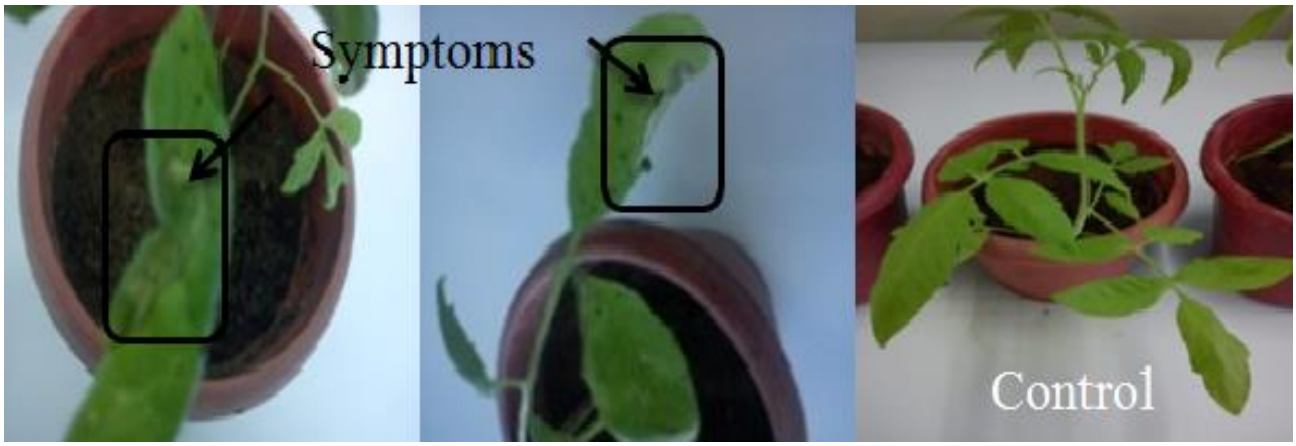

Figure 2. Pathogenicity test of *A. alternata* on tomato.

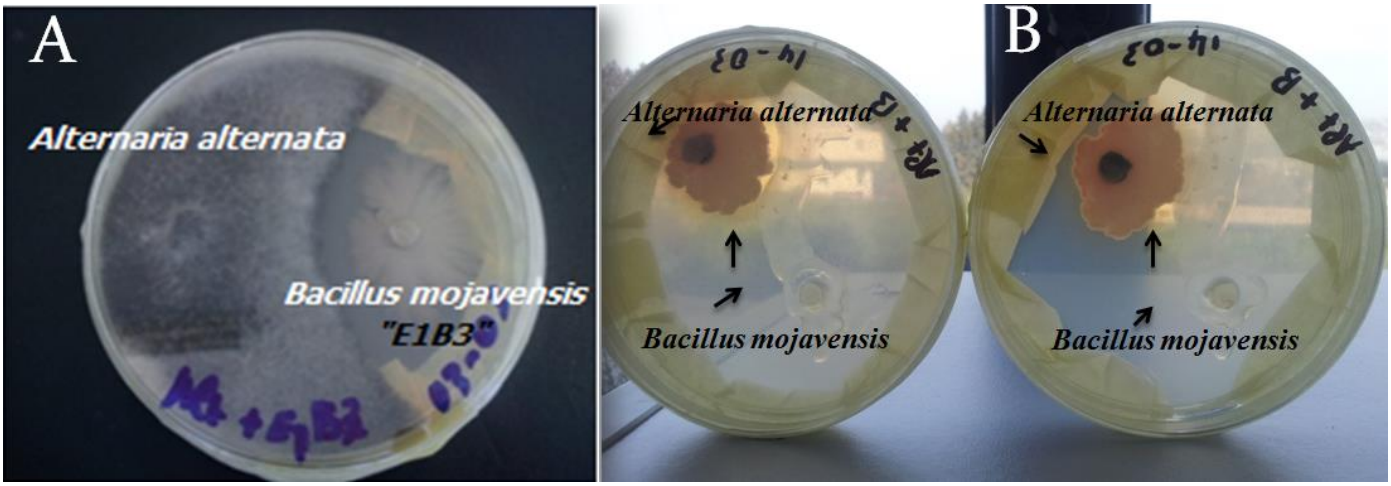

Figure 3. Inhibition of *A. alternata* by the bacterium (E1B3). The antagonism test was performed on PDA (A) and Czapek Dox Agar (B) plates. The bacteria and the fungi were inoculated at the same time and the antagonism was scored after 2-5 days of incubation at 28°C.

sulphate as sources of essential ions. The production of small conidia was observed when compared with the normal growth (Figure 4). The molecular identification of the screened strains (E1B3) by *16S r-RNA* sequences analysis showed that it is closely related to a *Bacillus subtilis* group including several species. However, *gyraseA* sequences provided more precise identification and classify it among members of *Bacillus mojavensis* (KC977492) species.

A relative antifungal performance against the same or other fungi were widely investigated previously by diverse *Bacillus* species in the case of *in- vitro* and *vivo* tests. In fact, for *in vitro* tests, Pengnoo et al. (2006) found that 16 isolates of *Bacillus* spp. had the ability to inhibit mycelia growth of *Rhizoctonia solani*, causal agent of leaf blight of bambara groundnut. Among these isolates, *Bacillus firmus* had the greatest activity in anti-microbial tests against *Rhizoctonia solani*. In the other hand, in the case of in *vivo tests*, some data can be presented as the control of early leaf spot of peanut (Kokalis et al., 1992), yam leaf spot (Michereff et al., 1994), grey mould of strawberries, and post-bloom fruit drop of citrus (Sonoda et al., 1996).

The growth inhibition ability of *B. mojavensis* on other plant pathogens (*Ambrosiella macrospora*, *Botrytis cinerea*, *Fusarium oxysporum*, *Fusarium moniliforme*, *Macrophomina phaseolina*, *Mucor rammanianus*, *Alternaria solani*, *Phytophthora meadii*, *Pythium aphanidermatum* and *Rhizoctonia solani*), was demonstrated in previous studies (Nair et al., 2002; Youcef-Ali et al., 2014). This showed the possibility of using *B. mojavensis* strain to manage the production of several crops sensible to the above cited fungi. It has been shown that the suppressive effect of *B. mojavensis* on *A. alternata* could also be correlated with secreting inhibitory molecules.

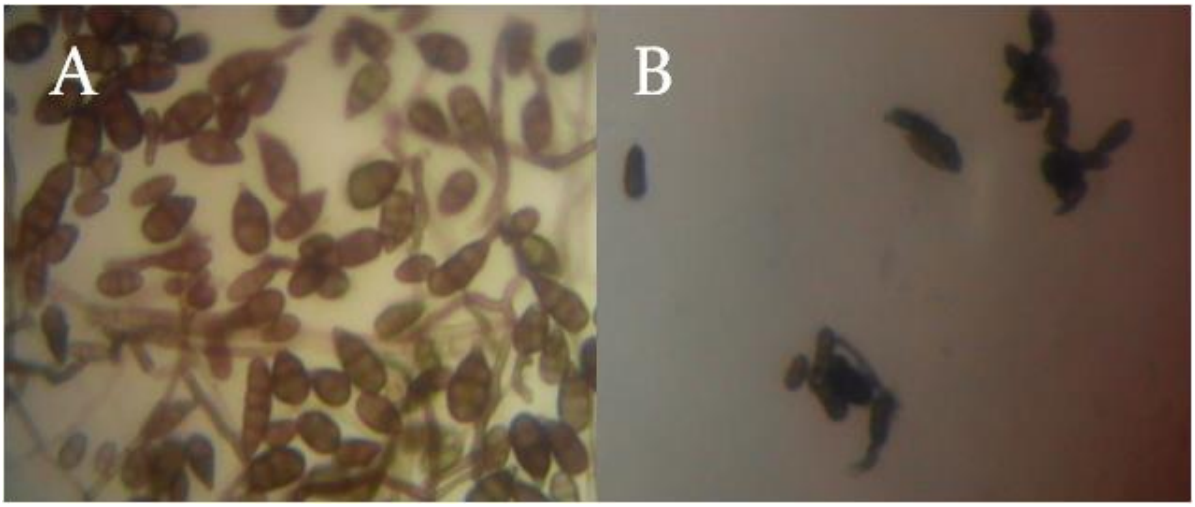

Figure 4. Effect of E1B3 on "intracellular and/or intercellular" growth areas of the pathogen *A. alternata.* (A) Fungus growth in absence of E1B3 and (B) in the presence of E1B3.

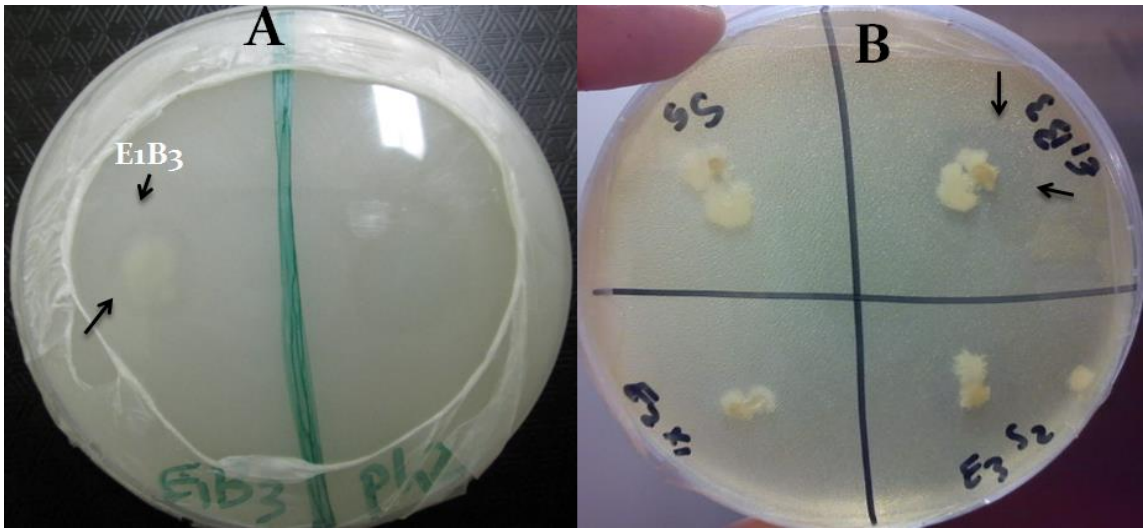

Figure 5. A) Detection of protease activity developed by *B. mojavensis* KC977492. B) Detection of lipase activity developed by *B. mojavensis* KC977492.

Cell-wall degrading enzymes and LPs production

A number of antifungal compounds, including polypeptides that interact with the fungal membrane, are produced by *Bacillus* species (Rocha et al., 2014). This process of fungal growth inhibition was attributed to many mechanisms, including the production of antimicrobial molecules, the secretion of hydrolytic enzymes, the competition for nutrients, or the combination of mechanisms (Compant et al., 2005; Rocha et al., 2014). Lipopeptides, that is, surfactin, iturin and fengycin, were described previously as major classes of *Bacillus* antibiotic peptides. These molecules have a great potential for diverse biotechnological applications (Peypoux et al., 1999; Ait Kaki et al., 2013). *B. subtilis* group including *B. mojavensis* is recognized as a safe organism (GRAS) by the United States Federal Drug Administration (FDA) (Sanders et al., 2003).

In this work, the screening of antagonist bacterium, *B. mojavensis* showed a negative result in the enzymatic activities test of chitinase, while, there is an important positive production of lipase and protease (Figure 5) with a clear halo on medium reaching 0.5 cm. On the other hand, it has been shown that *B. mojavensis* produce three LPs families, iturin, fengycin and surfcatin after interpreting mass spectra corresponding to mass

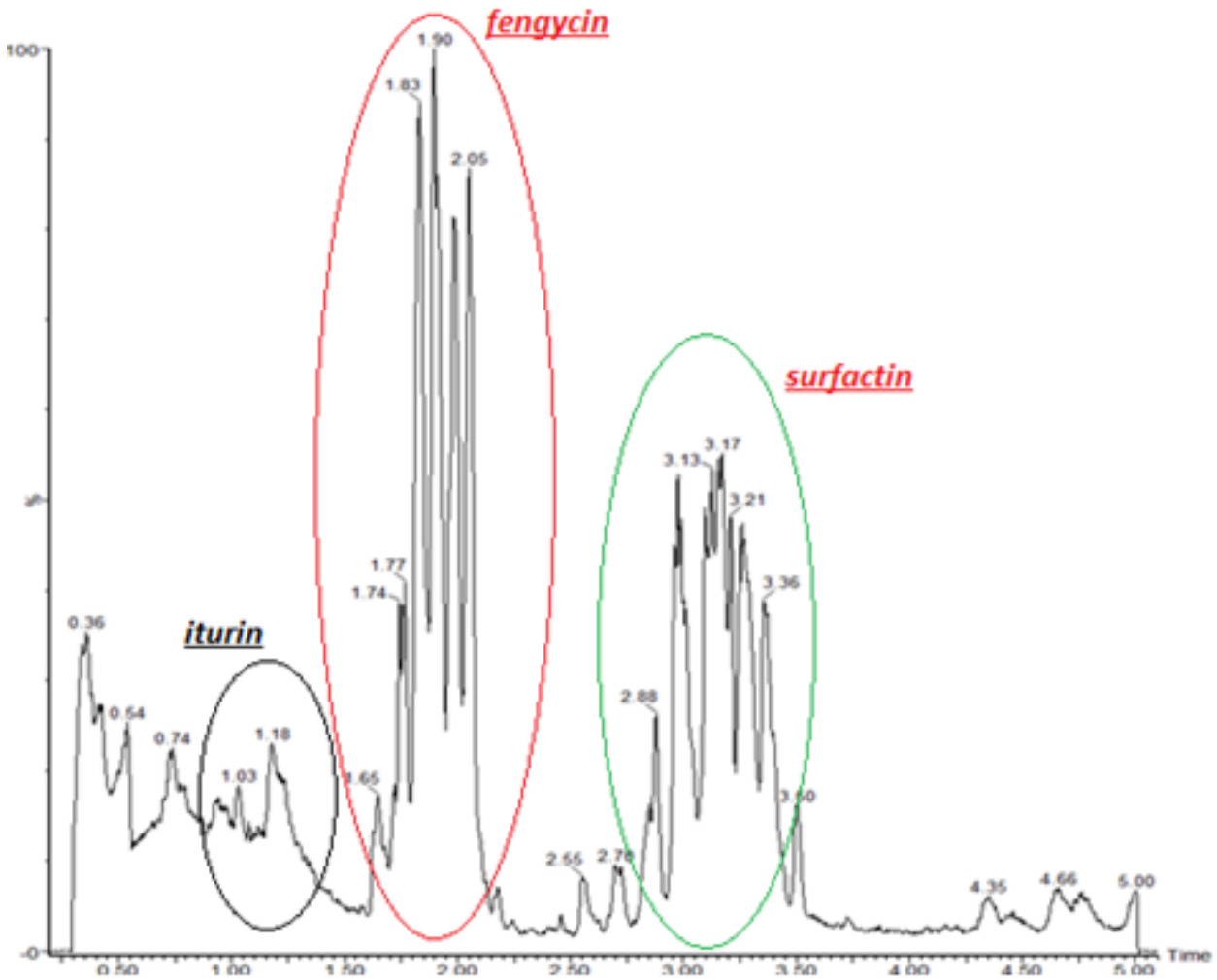

Figure 6. MS profile of *B. mojavensis*. MS analysis showing the production of three LPs families at different retention time, iturin (80s to 1.20 min), fengycin (1.7 to 2.2 min) and surfactin (2.7 to 3.40 min).

spectrometry (MS) peaks (Figure 6).

In comparison, Youcef-Ali et al. (2014) showed that *B. subtilis* and *B. mojavensis* produce enzymes such as cellulase and protease, but not chitinase in *B. mojavensis*. The same results were obtained in lipopetide production by members of *B. subtilis* group. In fact, Yu et al. (2002), Fernandes et al. (2007) and Ait kaki et al. (2013) reported that *B. amyloliquefaciens* B94, *B. subtilis* and *B. subtilis spizezenii* produced lipopeptides families' iturin, fengycin and surfcatin. The capacity of lipopetides production is restricted on members of *Baciillus subtilis* group. Indeed, *P. polymyxa* (18SRTS) did not produce any type of lipopeptides as mentioned by Ait kaki et al. (2013).

Flow cytometry analysis

The antagonistic effect of the *Bacillus* strain against *A. alternata* was investigated here and previously in other studies by dual culture technique (Ait Kaki et al., 2013). However, to the authors' best knowledge, the study of the antagonistic effect of *B. mojavensis* against *A. alternata* by using flow cytometry analysis was investigated for the first time in the present work. This technique has emerged as a high-resolution technology that supports the characterization of individual cell types within mixed populations. It provides an analysis of a large number of cells and can identify changes within a population and between different populations (O'Donnell et al., 2013). Carboxyfluorescein diacetate (CFDA) is a lipophilic, non-fluorescent dye, used primarily for the evaluation of a cellular enzymatic activity and for the fluorescence labeling. The ester bonds are hydrolyzed by enzymes with esterase activity, yielding the green fluorescent dye molecules because enzyme activity is needed for hydrolysis, and membrane integrity is required for the retention inside cells. It is supposed that viable cells

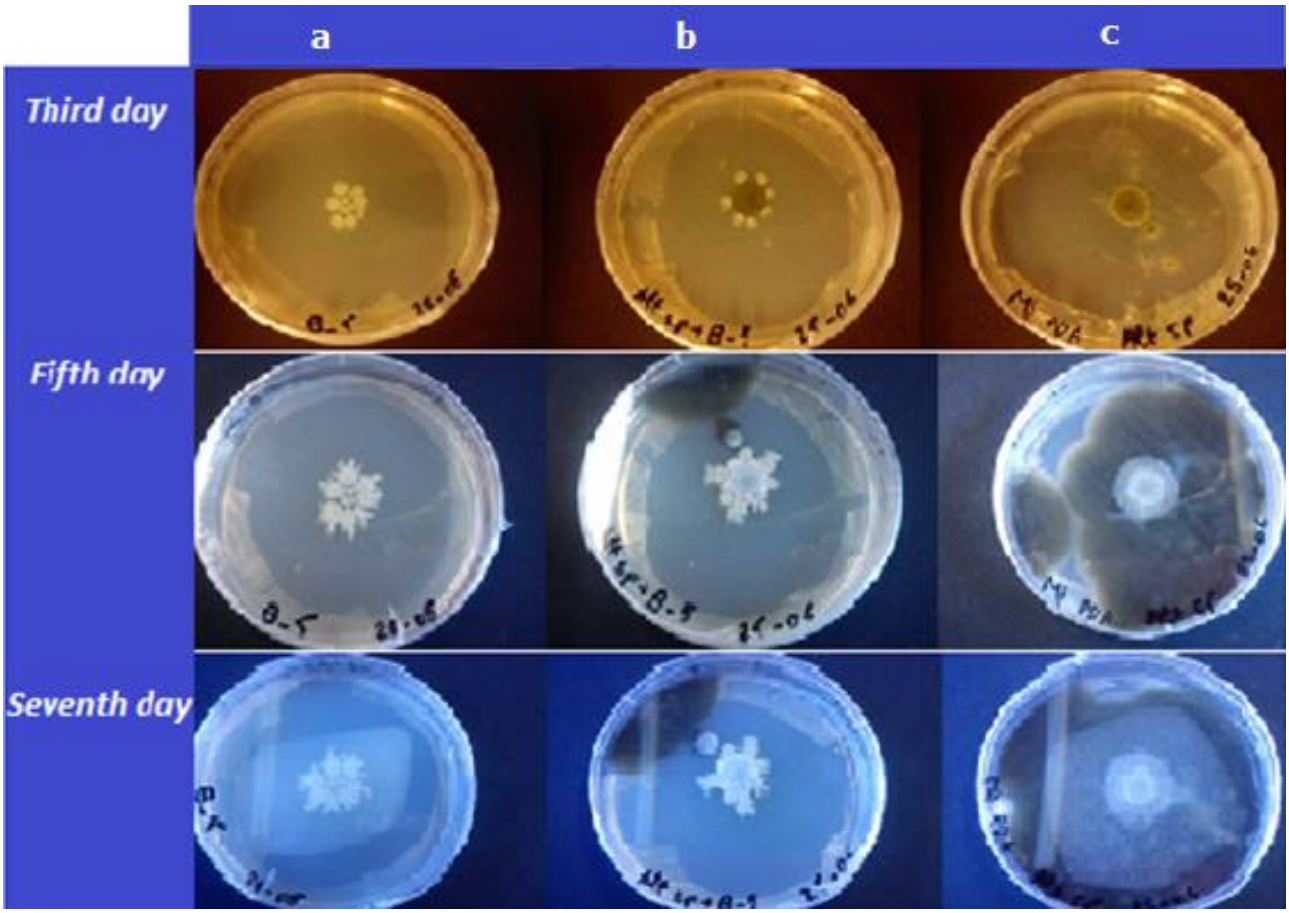

Figure 7. Effect of *B. mojavensis* on the mycelial growth of *A. alternata* during 7 days growth on potato dextrose agar (PDA). The effect in the third, fifth and seventh day are shown in this figure. (A) PDA plates inoculated with *B. mojavensis* strain, (B) PDA plates inoculated centrally by *B. mojavensis* and *A. alternata* strain and (C) PDA plates inoculated with *A. alternate.*

accumulate fluorescein (derivatives) but dead cells are not able to do so (Rotman and Papermaster, 1966).

In this study, the effect of *B. mojavensis* on *A. alternata* growth at intracellular and intercellular levels was carried by FCM analysis. The growth evolution of *B. mojavensis* and *A. alternata* centrally cultured on PDA plates showed a high inhibition of the fungus isolate by the bacterium *B. mojavensis* during the incubation time, from the third to the seventh day (Figure 7). Figure 8 shows a good microbial (*B. mojavensis* and *A. alternata* cultred on PDA plates) staining with a fluorescent product CFDA in FCM analysis. Furthermore, FCM profiles of dead and alive *B. mojavensis* and *A. alternata* cells was verified before launching analysis on PDA plates where fungal and bacterial strains were centrally cultured.

Before treating microbial preparations with heat (90°C during 12 min), the green fluorescent intensity (GFI) of alive cells reached 10^4 and 10^5 MH (megahertz), respectively in the case of *B. mojavensis* (Figure 9A) and *A. alternata* (Figure 10A). However, dead cells GFI of both of them was less important varying between 10^2 and 10^3 MH (Figure 9B and C). The evolution of green fluorescence intensity of *B. mojavensis, A. alternata* and *B. mojavensis-A. alternata* interaction plates from the third to the seventh day of time incubation was carried by FCM analysis.

After the 3rd day, the green fluorescence intensity of *B. mojavensis* reached its maximum at 10^4 MH, that of *A. alternata* reached 10^6 MH. However, this intensity was between 10^3 and 10^4 MH in the case of *B. mojavensis-A. alternata* interaction plates (Figure 11). After the 5th day, the green fluorescence intensity GFI of *B. mojavensis* decreased slightly until reaching 10^3 MH. Two peaks in 10^3 and 10^6 MH were observed in the case of *A. alternata*. However, the GFI was stable close to 10^3 MH in the case of *B. mojavensis-A. alternata* interaction (Figure 11). After seven days of incubation, approximate GFI values of the fifth day were obtained in *B. mojavensis and A. alternata* plates. Interestingly, GFI of microbial interaction plate increased reaching values between 10^4 and 10^5 MH.

In the control, the GFI values of *B. mojavensis* (Figure 9B,C) showed that not all cells were dead after 12 min at 90°C, this can be explained by the presence of spores

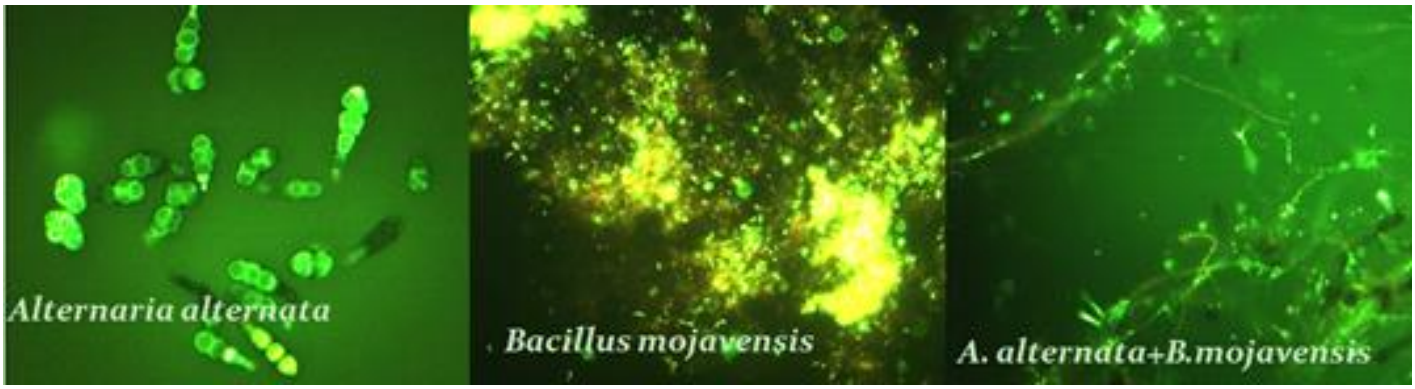

Figure 8. Interaction between *A. alternata* and *B.mojavensis* monitored by fluorescence microscopy. Cells were treated as described in the Materials and Methods section. Fungal cells were stained with CFDA (green fluorescence) microscopic observation showed in this figure was 7 days of interaction (GX 40).

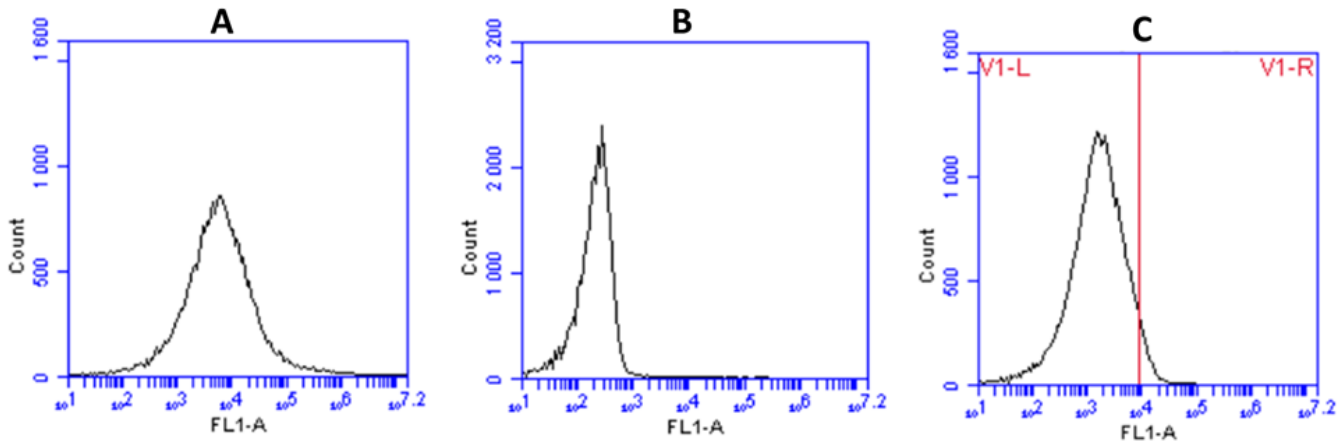

Figure 9. Flow cytometry profile of control, *B. mojavenisis* plates after 3 days of incubation. (A) bacterial cells alive before heat treatment (90°C during 12 min), (B) dead bacterial cells not colored with CFDA, after heat treatment, (C) bacterial dead cells colored with CFDA, after heat treatment.

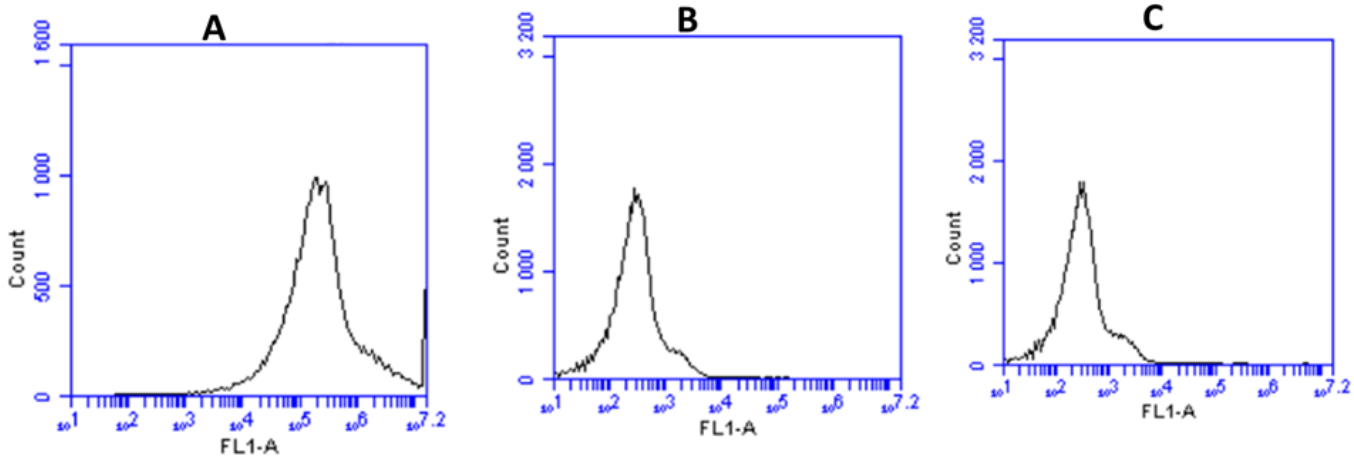

Figure 10. Flow cytometry profile of the control, *A. alternata* plates after 3 days of incubation. (A) fungal cells alive before heat treatment (90°C during 12 min), (B) dead fungal cells not colored with CFDA, after heat treatment, (C) dead fungal cells colored with CFDA, after heat treatment.

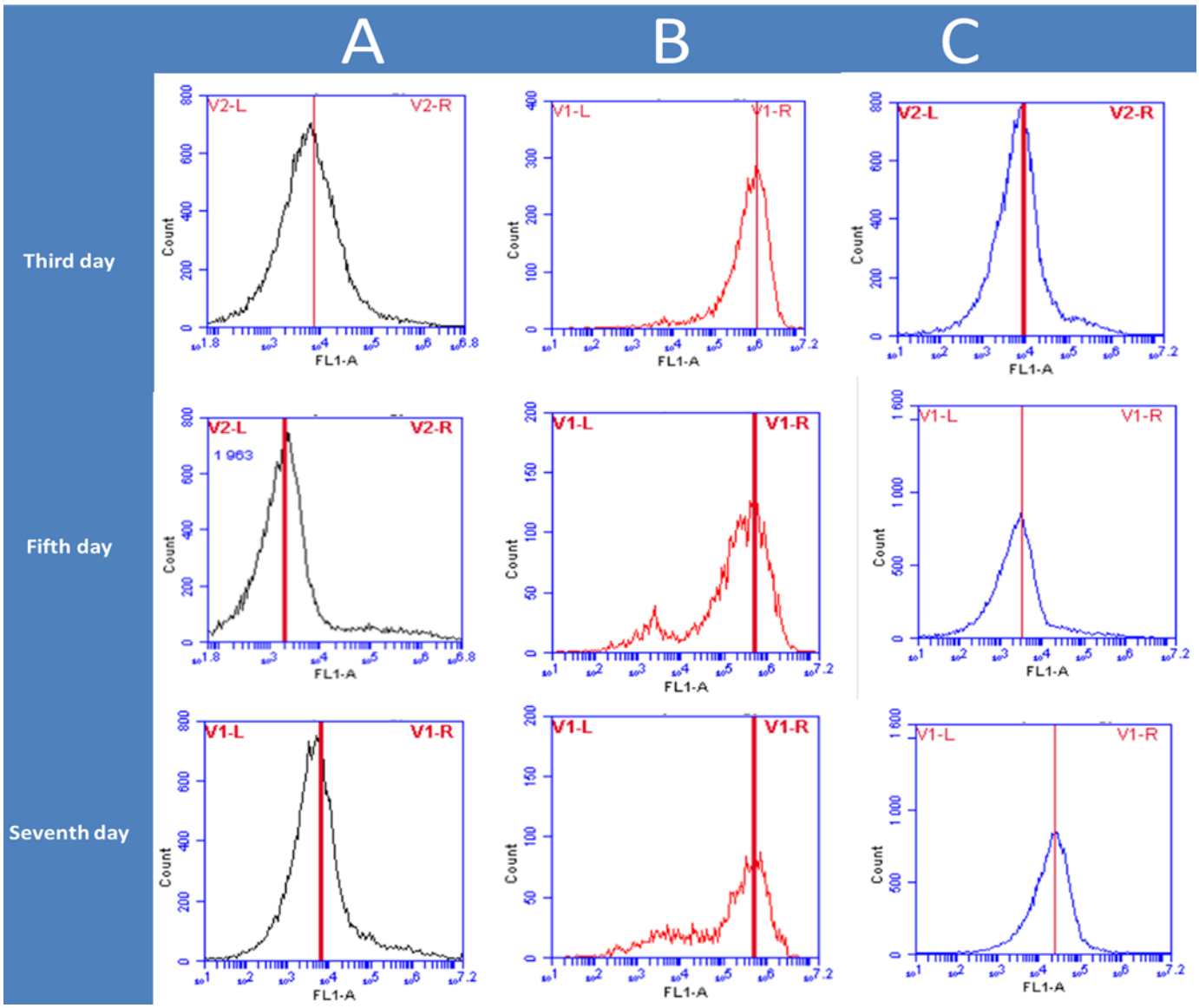

Figure 11. Flow cytometry profiles experimental of *B. mojavensis* (A), *A. alternata* (B), *B. mojavensis-A. alternata* interaction (C) after the third, fifth and the seventh day of incubation.

that are resistant to high temperatures (Rocha et al., 2014).

Cells of *A. alternata* are viable with a maximum GFI which reached 10^6 MH (Figure 11B) after the 3rd, 5th and 7th day, in comparison with the control, dead cells of *A. alternata*, which was 10^2 MH (Figure 10C). However, the GFI of the interaction between *A. alternata* and *B. mojavensis* was proximate to that of *B. mojavensis* which was between 10^3 and 10^4 MH, after the 3rd, 5th and 7th day (Figure 11A and C). These results confirm that *B. mojavensis* deteriorate fugal mycelium of *A. alternata.*

In fact, the comparison of GFI values of the FCM analysis (Figure 11) and the effect of *B. mojavensis* on *A. alternata* after the 3rd, 5th and 7th day on (PDA) plates (Figure 7) confirm the antifungal activity of *B. mojavensis* against *A. alternata.* Rocha et al. (2014) used flow cytometric for studying the interaction between *Fusarium verticillioides* and *Bacillus thuringiensis* Subsp. Kurstaki using Calcofluor White (CFW) and 7 aminoactinomycin (7-AAD). A reduction in cell numbers in the treated cultures on the third, fifth and seventh days during the FCM analysis was observed.

Fluorescence microscopy

The microscopic examination by fluorescence microscopy revealed an evident antagonistic effect in the

seventh day, in comparison with the control, *A. alternata* culture (Figure 8). The results are the same with that of Rocha et al. (2014) on interaction between *F. verticillioides* and *B. thuringiensis* subsp*. kurstaki*.

Conclusion

In conclusion, the FCM method results showed interesting antagonistic effect of *B. mojavensis* on *A. alternata* while additional green house and field experiments on tomato plants are required for the possibility of using it as a biocontrol agent.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was financially supported by University Mentouri of Constantine. The authors thank Wallon Center of Industrial Biology (CWBI) for allowing them work in the laboratories of bioindustry.

REFERENCES

Agrios GN (1997). Plant pathology, 3rd edition. Academic Press, New York. U.S.A.

Ait Kaki A, Kacem-Chaouche N, Dehimat L, Milet A, Youcef-Ali M, Ongena M, Thonart P (2013). Biocontrol and Plant Growth Promotion Characterization of *Bacillus* Species Isolated from *Calendula officinalis* Rhizosphere. Indian J. Microbiol. 53(4):447-452.

Aneja KR (2003). Experiments in Microbiology Plant Pathology and Biotechnology, Fourth Edition, New Age International (P) Ltd., Publishers, New Delhi. P 320.

Baysal O, Lai D, Xu HH, Siragusa M, kan CM (2013). A proteomic approach provides new insights into the control of soil-borne plant pathogens by *Bacillus* species. PLoS One 8:e53182.

Beneduzi A, Peres D, Costa DPB, Zanettini MHB (2008). Genetic and phenotypic diversity of plant growth- promoting bacilli isolated from wheat fields in southern Brazil. Res. Microbiol. 159:244-250.

Berg G (2009). Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Appl. Microbiol. Biotechnol. 84:11-18.

Boyd AR, Gunasekera TS, Attfield PV, Simic K, Vincent SF, Veal DA (2003). A flowcytometric method for determination of yeast viability and cell number in a brewery. FEMS Yeast Res. 1(3):11-16.

Bunthof CJ, Braak VDS, Breeuwer P, Rombouts FM, Abee T (2000). Fluorescence assessment of *Lactococcus lactis* viability. Int. J. Food Microbiol. 55:291-294.

Compant S, Duffy B, Nowak J, Clement C, Barka EA (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl. Environ. Microbiol. 71:4951-4959.

Czechowska K, Johnson DR, Meer VDJR (2008). Use of flow cytometric methods for single-cell analysis in environmental microbiology. Curr. Opin. Microbiol. 3(11):205-212.

Emmert EAB, Handelsman J (1999). Biocontrol of plant disease: a Gram positive perspective. FEMS. Microbiol. Lett. 171:1-9.

Etcheverry MG, Scandolara A, Nesci A, Ribeiro MSVB, Pereira P (2009). Biological interactions to select biocontrol agents against toxigenic strains of *Aspergillus flavus* and *Fusarium verticillioides* from maize. Mycopathologia 167:287-295.

Fernandes PAV, Arruda DIR, Santos DAFAB, De, Araujo AA, Maior AMS, Ximenes EZ (2007). Antimicrobial activity of surfactants produced by *Bacillus subtilis* R14 against multidrug-resistant bacteria. Braz. J. Microbiol. 38:704-709.

Fiddaman PJ, Rossall S (1993). The production of antifungal volatiles by *Bacillus subtilis*. J. Appl. Bacteriol. 74:119-126.

Flint S, Drocourt JL, Walker K, Stevenson B, Dwyer M, Clarke I, McGill D (2006). A rapid, two-hour method for the enumeration of total viable bacteria in samples from commercial milk powders and whey protein concentrate manufacturing plants. Int. Dairy J. 16:379-384.

Gauthier C, St-Pierre Y, Villemur R (2002). Rapid antimicrobial susceptibility testing of urinary tract isolates and samples by flow cytometry. J. Med. Microbiol. 51(3):192-200.

Gilbert J, Haber S (2013). Overview of some recent research developments in *Fusarium* head blight of wheat. Can. Plant Pathol. 35:149-174.

Hewitt CJ, Caron NVG (2001). An industrial application of multiparameter flow cytometry: Assessment of cell physiological state and its application to the study of microbial fermentations. Cytometry 3(44):179-187.

Izumi S, Aranishi F (2004). Relationship between gyrA mutations and quinolone resistance in *Flavobacterium psychrophilum* isolates. Appl. Environ. Microbiol. 70:3968-3972.

Jacques P, Hbid C, Destain J, Razafindralambo H, Paquot M, Pauw DE, Thonart P (1999). Optimization of biosurfactant lipopeptides production from *Bacillus subtilis* S499 by Plackett–Burman design. Appl. Biochem. Biotechnol. l77:223-233.

Knox OGG, Killham K, Leifert C (2000). Effects of increased nitrate availability on the control of plant pathogenic fungi by the soil bacterium *Bacillus subtilis.* Appl. Soil. Ecol. 15:227-231.

Kokalis BN, Backman PA, Kabana R, Ploper LD (1992). Potential for biological control of early leaf spot of peanut using *Bacillus cereus* and chitin as foliar amendments. Biol. Control 2:321-328.

Larpent JP, Larpent GM (1985). Manuel pratique de microbiologie. Hermann 21, France. P 230.

Leelasuphakul W, Sivanunsakul P, Phongpaichit S (2006). Purification, characterization and synergistic activity of 1, 3-glucanase and antibiotic extract from an antagonistic *Bacillus subtilis* NSRS 89-24 against rice blast and sheath blight pathogens. Enzyme Microb. Technol. 38:990-997.

Michereff SJ, Silveira NSS, Reis A, Mariano RLR (1994). Epiphytic bacteria antagonistic to *Curvularia* leaf spot of yam. Microbiol. Ecol. 28:101-110.

Müller S, Caron NVG (2010). Functional single cell analyses: Flow cytometry and cell sorting of microbial populations and communities. FEMS. Microbiol. Rev. 34(4):554-587.

Nair JR, Singh G, Sekar V (2002). Isolation and characterization of a novel *Bacillus* strain from coffee phyllosphere showing antifungal activity. J. Appl. Microbiol. 93:772-780.

Ngueфack J, Budde BB, Jakobsen M (2004). Five essential oils from aromatic plants of Cameroon: their antibacterial activity and ability to permeabilise the cytoplasmic membrane of *Listeria innocua* examined by flow cytometry. Lett. Appl. Microbiol. 39:395-400.

Nihorimbere V, Pascal K, Ernest S (2013). Isolation of *Bacillus* strains from the rhizosphere of Tomato and their *in vitro* antagonistic effects against phytopathogenic fungi. Glob. Adv. Res. J. Microbiol. 3(2):65-71.

O'Donnell EA, Ernst DN, Hingorani R (2013). Multiparameter flow cytometry: advances in high resolution analysis. Immune Netw. 13:43-54.

Palazzini JM, Ramirez ML, Torres AM, Chulze SN (2007). Potential biocontrol agents for *fusarium* head blight and deoxynivalenol production in wheat. Crop Prot. 26:1702-1710.

Pamela C, Ernesto OO, Esperanza MR, Doris Z (2010). Characterization of *Bacillus* isolates of potato rhizosphere from Andean soils of Peru and their potential PGPR characteristic. Braz. J. Microbiol. 41:899-906.

Pengnoo A, Wiwattanapattapee R, Chumthong A, Kanjanamaneesathian M (2006). Bacterial antagonist as seed treatment to control leaf blight disease of bambara groundnut (*Vigna subterranean*). World J.

Microbiol. Biotechnol. 22:9-14.
Peralta IE, Knapp S, Spooner DM (2005). New species of wild tomatoes (Solanum section Lycopersicon: Solanaceae) from Northern Peru. Syst. Bot. 30:424-434.
Petit JM, Gay DM, Ratinaud MH (1993). Assessment of fluorochromes for cellular structure and function studies by flow cytometry. Biol. Cell 78:1-13.
Peypoux F, Bonmatin JM, Wallach J (1999). Recent trends in the biochemistry of surfactin. Appl. Microbiol. Biotechnol. 51:553-563.
Pinchuk IV, Bressollier P, Sorokulova IB, Verneuil B, Urdaci MC (2002). Amicoumacin antibiotic production and genetic diversity of *Bacillus subtilis* strains isolated from different habitats. Res. Microbiol. 153:269-276.
Rault A, Béal C, Ghorbal S, Ogier JC, Bouix M (2007). Multiparametric flow cytometry allows rapid assessment and comparison of lactic acid bacteria viability after freezing and during frozen storage. Cryobiology 1(55):35-43.
Rocha LO, Tralamazza SM, Reis GM, Rabinovitch L, Barbosa CB (2014). Multi-Method Approach for Characterizing the Interaction between *Fusarium verticillioides* and *Bacillus thuringiensis Subsp. Kurstaki.* PLoS ONE 4(9):e92189.
Rotman B, Papermaster BW (1966). Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proc. Natl. Acad. Sci. USA. 55:134-141.
Sanders ME, Morelli L, Tompkins TA (2003). Sporeformers as human probiotics: *Bacillus, Sporolactobacillus, and Brevibacillus*. Compr. Rev. Food Sci. Food Safe. 2:101-110.
Snoussi SA (2009). Basic study on tomato in Algeria. Regional Programme for Integrated Pest Management for the Middle East.www.ipm-neareast.com
Sonoda RM, Guo ZT, Nemec S (1996). Effect of spray applications of *Bacillus subtilis* on postbloom fruit drop of citrus. Phytopathology 86:S52-S53.
Tanaka S, Yamaguchi N, Nasu M (2000). Viability of *Escherichia coli* O157:H7 in natural river water determined by the use of flow cytometry. J. Appl. Microbiol. 88:228-236.
Thom C, Church M (1926). The Aspergilli. P 39.
Toure Y, Ongena M, Jacques P, Guiro A, Thonart P (2004). Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple. J. Appl. Microbiol. 96:1151-1160.
Walberg M, Gaustad P, Steen HB (1996). Rapid flow cytometric assessment of mecillinam and ampicillin bacterial susceptibility. J. Antimicrob. Chemother. 37(6):1063-1075.
Xue AG, Voldeng HD, Savard ME, Fedak G, Tian X (2009). Biological control of *fusarium* head blight of wheat with *Clonostachys rosea* strain ACM941.Can. J. Plant. Pathol. 31:169-179.
Youcef-Ali M, Kacem-Chaouche N, Dehimat L, Bataiche I, Kara-Ali M, Cawoy H, Thonart P (2014). Antifungal activity and bioactive compounds produced by *Bacillus mojavensis* and *Bacillus subtilis*. Afr. J. Microbiol. Res. 8(6):476-484.
Yu GY, Sinclair JB, Hartman GL, Bertagnolli BL (2002). Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. Soil. Biol. Biochem. 34:955-963.

African Journal of Biotechnology

Related Journals Published by Academic Journals

- *Biotechnology and Molecular Biology Reviews*
- *African Journal of Microbiology Research*
- *African Journal of Biochemistry Research*
- *African Journal of Environmental Science and Technology*
- *African Journal of Food Science*
- *African Journal of Plant Science*
- *Journal of Bioinformatics and Sequence Analysis*
- *International Journal of Biodiversity and Conservation*

academicJournals