academicJournals

Vol. 7(47), pp. 5314-5323, 28 November, 2013
DOI: 10.5897/AJMR2013.6029
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

A novel *Alcaligenes faecalis* antibacterial-producing strain isolated from a Moroccan tannery waste

Ilham Zahir*, Abdellah Houari, Wifak Bahafid, Mohammed Iraqui and Saad Ibnsouda

Laboratory of Microbial Biotechnology, Department of Biology, Faculty of Sciences and Technology, University Sidi Mohamed Ben Abdellah, BP 2202, Road of Immouzer, Fez, Morocco.

Accepted 6 November, 2013

Drug resistance of many harmful bacteria still represents a real public health problem. This is why the development of new bioactive substance becomes urgent. To reach our goal, a screening of bioactive substance produced by microorganism, was undertaken. In this work, we reported data on a bacterial strain isolated from the tannery of Fez (Morocco) that showed a broad antagonistic effect against a group of Gram-negative and positive bacteria especially *Mycobacterium smegmatis*. The antibacterial compounds producer, BW1 was identified as *Alcaligenes faecalis* on the basis of phenotypic characteristics, biochemical analyses and PCR amplification of 16 S ribosomal RNA gene followed by sequencing (100% of homology). The antibacterial compounds were synthesized during the exponential growth phase of *A. faecalis*. The antibacterial compounds were not affected following heat treatment and protolytic enzymes that indicated the non-proteinaceous nature of the active agents. The bacterial extract was assayed for cellular toxicity to fresh human erythrocytes and found to have no hemolytic effect.

Key words: Bacterial resistance, antibacterial compounds, *Alcaligenes faecalis*.

INTRODUCTION

Bacterial resistance to antibiotics poses a serious challenge to the prospect of chemotherapy, because of traditional antibiotics and its derivatives are becoming nonfunctional (Sengupta and Chattopadhyay, 2012). The whole world is thus confronted with a looming drug crisis which has motivated the pursuit of new antibiotic compounds with novel mechanisms of action (Sengupta and Chattopadhyay, 2012).

Natural products from bacteria have served as lead compounds for the development of pharmaceutical drugs that are widely used to fight bacterial infections (Bredholdt et al., 2007). Thus, this present study highlights an antibacterial compounds producing bacterium isolated from the tannery of Fez (Morocco) which is *Alcaligenes faecalis*.

The genus *Alcaligenes* is known among the bacteria having antagonistic activity (Austin, 1989; Bernan et al., 1997; Jayanth et al., 2001). Basically, *Alcaligenes denitrificans* had an algicidal activity on *Microcrocystis spp* (Manage et al., 2000) and *Alcaligenes xylosoxydans* was considered as a potential antifungal biocontrol (Vaidya et al., 2001). Several investigations had found out that *A. faecalis* displayed an antifungal activity against *Aspergillus niger*, *Paecilomyces variotii, Candida albicans* (Li et al., 2007) and *Fusarium oxysporum* (Honda et al., 1998; Santos et al., 2011). This microorganism, was also able to inhibit the growth of many bacteria such as *Bacillus subtilis* (Li et al., 2007), *Staphylococcus aureus* (Li et al., 2008), *Pseudomonas aeruginosa, Mycobacterium avium* and *Mycobacterium*

*Corresponding author. E-mail: ilham_biologie@hotmail.fr. Tel: 00212665615583. Fax: 00212535510269.

tuberculosis (Bacic and Yoch, 2001). However, no research has reported the inhibitory effect of *A. faecalis* against *Erwinia chrysanthemi.*

There are few studies demonstrating antibiosis effect of isolated strains from tannery waste like in Rai et al. (2009) research. It was found that a bacteriocin produced by *Enterococcus faecium* was antagonistic to several human pathogens including *Listeria*, *Aeromonas, Staphylococcus* and *Salmonella*. Moreover, the antibacterial effect of *A. faecalis* has never been elucidated by any other investigations from Moroccan ecological zones.

Thus, the objectives of this work include: (a) screening for an antibacterial compounds-producing bacteria, (b) identifying strains on the basis of Gram stain, biochemical characteristics and PCR followed by DNA sequencing of 16S ribosomal RNA gene, (c) evaluating antimicrobial activity of the isolated bacterium against a wide range of Gram positive and negative bacteria and (d) partially characterizing the secreted substances.

MATERIALS AND METHODS

Bacterial strains and media

Mycobacterium smegmatis MC² 155 is a non pathogenic atypical strain with a generation time of approximately 3 h (Grosset et al., 1989). *M. aurum* A^+ is non pathogenic bacterium with a generation time of approximately 6 h. This strain is used as a model to evaluate the effect of active substances on the growth of *M. tuberculosis* (Chung et al., 1995). The mycobacteria were kindly provided by Dr. Suzana David (Centro de Tuberculose e Micobactérias Instituto Nacional de Saúde Dr. Ricardo Jorge Delegação do Porto, Portugal); *Staphylococcus aureus* (Hamadi and Latrache, 2008); *Bacillus subtilis* ILP 142B (Hamadi and Latrache, 2008); *Pseudomonas aeruginosa* (Hamadi and Latrache, 2008); *Escherichia coli* Dh5α (Microbial biotechnology laboratory of Techniques and Sciences Faculty, Fès); *Erwinia chrysanthemi* 3937 (Hassouni et al., 1999). This bacterium was friendly provided by Dr. Hassouni (LCB-CNRS-Marseille). These strains were propagated in Luria-Bertani (LB) at 37°C or at 30°C for *E. chrysanthemi*.

The isolate was stored at -70°C in LB broth supplemented with 25% glycerol. Throughout the experiments, strains were sub-cultured every week on agar media and held at 4°C.

Different media in broth or on agar plates were used including respectively Luria-Bertani medium containing 10 g of peptone, 5 g of yeast extract, 10 g of NaCl per liter of distilled water and YPG medium containing 20 g of peptone, 10 g of yeast extract, 20 g glucose, 60 µg/ml of ampicillin and 30 µg/ml of kanamycin per liter of distilled water (Sambrook et al., 1989).

Screening and isolation of microorganism

Samples were collected from the tannery of Fez Morocco and treated independently according to the method followed by Hassi et al. (2007). Colonies that showed clear halos of inhibition against *M. smegmatis* were picked up and transferred to LB agar plates; these were incubated at 37°C and stored at 4°C for later assays.

Anti-mycobacterial activity assay

Anti-mycobacterial activity was performed by two different methods, that is, (a) agar-well diffusion assay as was led by Muriana and Klaenhammer (1991). In this method, inhibition zone around each well was evaluated by measuring its diameter. (b) A modified spot-on-lawn assay where a colony of the isolated strain was spotted onto the surface of LB agar plates which had been already spread with a broth culture of the indicator microorganism *M. smegmatis*. In both cases, plates were incubated at 37°C for 24 h and the antimycobacterial activity was detected by the observation of inhibition area surrounding the test strains (Tagg et al., 1976). These assays were done three times and they were also carried out to evaluate the inhibitory activity of *E. coli* Dh5α used as a control.

Identification of antibacterial compounds-producing strains

Antibacterial compounds producing strain was examined for cellular morphology and Gram characteristics. The biochemical identify-cation was also performed according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Furthermore, the isolate was identified by molecular methods. These comprise 16S ribosomal DNA (rDNA) gene amplification by PCR and sequencing. The PCR amplification was performed with universal primers RS16 (5' TACGGCTACCTTGTTACGACTT 3') and fD1 (5' AGAGTTTGATCCTGGCTCAG 3') targeted against regions of 16S rDNA (Weisberg et al., 1991). The amplification protocol was as described by Zahir et al. (2011).

PCR amplicons were purified and sequenced using the Big Dye Terminator with primers (reverse and forward) while automated sequencing of both strands of the PCR products was done on a BIOSYSTEME 3130 automated gene sequencer (Sanger et al., 1977).

Identification analysis was realized by an alignment of consensus sequence of the 16S rDNA genes collected in an international database (Genebank) present at the NCBI website located at http://www.ncbi.nlm.nih.gov/BLAST. The results were then expressed in percentage of homology between the submitted sequence and the sequences resulting from the database.

Inhibitory spectrum of isolated strain

Spot-on-lawn assay was used to evaluate the inhibitory spectrum of the isolated culture strain. Gram-positive and negative bacteria were assayed comprising *M. aurum, Staphyloccus aureus*, *P. aeruginosa, B. subtilis*, *E. coli* Dh5α and *E. chrysanthemi*.

Inhibition was scored based on an abstract scale as follows: -, No inhibition; +, presence of inhibition's zone. The assay was repeated three times.

Antibiotic extraction

The bioactive substances were extracted by ethyl acetate as was described by Hassi et al. (2012). This assay was triplicate using the indicator strains *M. smegmatis, E. coli, P. aeruginosa, B. subtilis* and *S. aureus*. It was also carried out to evaluate the inhibitory activity of ethyl acetate crude extract of *E. coli* Dh5α which was used as a negative control.

Determination of the kinetics

Synthesis of antibacterial compounds was monitored during the growth cycle by growing the culture of the producer strain on LB broth under shaking condition. This was carried out as was previously described (Abo-Amer, 2007).

It is worth mentioning that the indicator bacteria used for the rest of the steps is *M. smegmatis*.

Physico-chemical characterization

Thermostability

To check the thermal stability, acetyl ethyl extract of the bacterial cells was exposed to 121°C (20 min), 100°C (15 min), 80°C (30 min), 37°C (3 h) and 4°C (six months) and subsequently the activity was checked as previously described (Iqbal et al., 2001; Thangam and Rajkumar, 2006).

Effect of enzymes

Pepsin (Sigma), proteinase K (Sigma) trypsin (Sigma) and α-chymotrypsin (Merck) were tested for their proteolytic activity on the crude acetyl ethyl extract of the antibacterial compounds from the bacterial strain.

The assay was performed at a final concentration of 1 mg/ml respectively at pH 3 and 7. Samples with and without enzymes were held at 37°C for 3 h and the remaining activity was determined by well-diffusion assay. Extracts not treated by proteases were used as controls (Ahmad and Rasool, 2003; Noonpakdee et al., 2003; Sifour et al., 2012).

Determination of toxicity of the extract on human erythrocytes

The hemolytic effect of the extract on red globules was evaluated using the method of washed erythrocytes with slight modifications (Roopan and Khan, 2009; Khan et al., 2011). Blood samples were collected in tubes with anticoagulant (Ethylenediaminetetraacetic acid), then 1 ml of blood was taken and washed three times with sterile saline solution of NaCl 0.9%. After each wash, the cells were centrifuged at 1500 rpm for 5 min and the supernatant was removed. The final pellet was diluted 1/100 in 0.9% NaCl solution and then volumes of 100 µl were removed and amalgamated respectively with different volumes of *A. faecalis* extract (20,100 and 200 µl). These preparations were supplemented subsequently with 0.9% NaCl having a final volume of 1.25 ml. After 4 h incubation at 37°C, the mixtures were centrifuged for 5 min at 1300 rpm and the supernatant was finally used to measure the absorbance of hemoglobin released at 540 nm. Erythrocytes were completely damaged after treatment with distilled water and the observed optical density was equivalent to 100% lysis (positive control), while the negative control matched 100 µl of blood dissolved in 1150 µl of saline NaCl 0.9%.

RESULTS AND DISCUSSION

Screening and isolation of microorganism

The screening of bacteria isolated from the tannery of Fez morocco showed six isolates having inhibitory properties by agar diffusible metabolites against *M. smegmatis*. After that, this anti-mycobacterial activity was confirmed by both spot-on-lawn assay and agar-well diffusion assay. Between the isolates, BW1 was the bacterium that showed the largest diameter of inhibition of about 12 ± 2 mm (Figure 1A and Table 1).

Identification of antibacterial compounds-producing strains

Morphologically, pure colonies of the bacterial strain BW1 were circular, low-convex, about 2 mm in diameter, smooth, shining and entire. It was a Gram-negative, short-rod or coccobacillary bacterium, arranged singly with an optimum temperature for growth at 30°C and no special pigments were produced, while growth was not allowed in 6.5% NaCl. Biochemically, the results are presented in Table 2.

The results collectively satisfy the criteria for the genus *Alcaligenes* given by Bergey's manual of determinative bacteriology: 0.5 to 0.6 by 2.0 µm in diameter, motile by means of one to eight peritrichous flagella, obligatory aerobic and carbohydrates are usually not utilized (Holt et al., 1994). Also, the results collectively corroborate with the findings of other studies (Coenye et al., 2003; Berkhoff and Riddle, 1984; Kiyohara et al., 1982; Pichinoty et al., 1978; Hendrie et al., 1974; Rarick et al., 1978; Kiredjiani et al., 1986; Lapage et al., 1973; Bacic and Yoch, 2001).

In the other hand, PCR amplification of the 16S rRNA gene with the fD1 and RS16 primers allowed the amplification of a DNA fragment of approximately 1.5 kb, as previously reported in the literature (Weisberg et al., 1991). The primers were shown to be able to amplify the 16S rRNA gene from eubacteria (Weisberg et al., 1991).

After DNA sequencing, sequences obtained with RS16 and fD1 primers were 414 and 435 bp, respectively.

BlastN search showed that the nucleotide sequence of 16S rDNA gene of the isolated strain BW1 had a homology of 100% to that of *A. faecalis*. The sequence was deposited in European Nucleotide Archive with the accession number HG737341. According to the criteria defined by Drancourt and collaborators (2000), the bacterial strain BW1 belonged to *A. faecalis* strain.

Regardless of its morphology, cultural appearance, and physiologic and biochemical characteristics mentioned above, together with the phylogenetic analysis (Figure 2), the strain BW1 was preliminary identified as *A. faecalis*. Based on these data, we assigned our strain as *A. faecalis* strain BW1.

Previously, *A. faecalis* had been isolated from tannery effluents as chromium resistant bacterium (Shakouri et al., 2010), but until now, no described antibacterial actives substances producer bacterium has been related to *A. faecalis* from Moroccan biotopes.

Spectrum activity

Spot-on-lawn assay was performed to assess the antagonistic activity of *A. faecalis* against indicator strains including *M. aurum, S. aureus*, *B. subtilis*, *E. coli* Dh5α, *P. aeruginosa* and *E. chrysanthemi*. The bacterium showed an antimicrobial activity against all tested bacteria (Figure 1). These results demonstrated that *A. faecalis* inhibit the growth of the indicator strains through the diffusion of antibacterial compounds into the medium.

The genus *Alcaligenes* is known among the bacteria showing antagonistic activity (Austin, 1989; Bernan et al.,

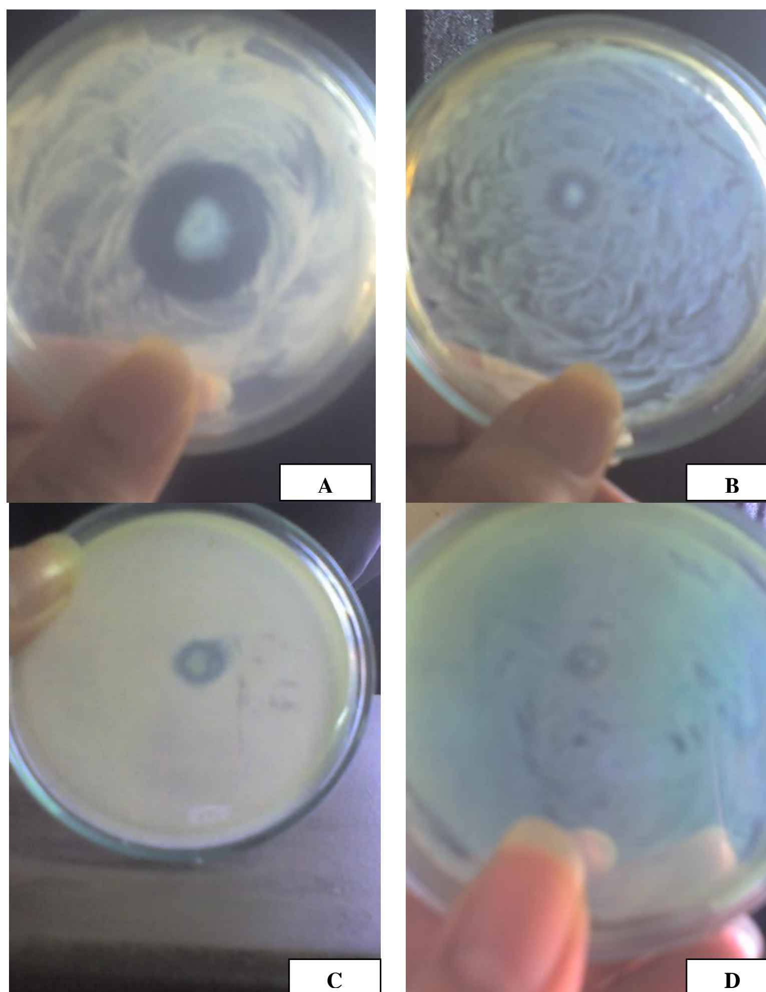

Figure 1. Inhibition zone of *A. faecalis* against *M. smegmatis* (A), *E. coli* (B), *B. subtilis* (C) and *P.aeruginosa* (D).Spot-on-lawn assay was performed to assess the antagonistic activity of *A. faecalis* against indicator bacteria. After incubation, the antibacterial effect was detected by the observation of inhibition area.

Table 1. Anti-mycobacterial activity assay of the isolates.

Isolate	Inhibition by spot-on-lawn assay	Diameter of inhibition halo by agar-well diffusion assay (mm)
Alpha	+	8 ± 1
CHP	+	8 ± 0.5
E1S5	+	10 ± 1.5
KI	+	4 ±1
L4	+	4 ±0.5
BW1	+	12±2
E. coli Dh5α (control)	-	0

Relative activity of the isolated strains was measured by both well-diffusion agar test and spot-on-lawn assay against *M. smegmatis*. (-): no inhibition; (+): inhibition.

1997; Jayanth et al., 2001). In fact, *A. piechaudii* had previously been shown to produce antibiotics and it was also able to inhibit mycelial growth and conidial germination of *Helminthosporium solani* (Assis et al., 1998;

Table 2. Biochemical analysis of the strain BW1.

Biochemical characteristic	Isolate BW1
Oxidase test	+
Catalase test	+
Citrate test	+
Caprate test	+
Ortho-nitrophenol test	-
Ornithine decarboxylase	-
Urea splitting	-
Nitrate reduction	-
Indole production	-
Voges-Proskaue test	-
H_2S production	-
Hydrolysis of:	
Starch	-
Gelatin	-
Esculin	-
Arginine	-
Acid production from carbohydrates:	
Glucose	-
Arabinose	-
Sucrose	-
Rhamnose	-
Maltose	-
Melibiose	-
Inositol	-
Mannitol	-
Sorbitol	-
N-acetyl-glucosamine	-
Amygdalin	-
Adipate	-
Tryptophan	-
Lysine	-

+, Positive reaction; -, negative reaction.

Martinez et al., 2006). While *A. faecalis* displayed a wide spectrum of activity against seed and soilborne fungi such as *Alternaria alternate, Cladosporium cladosporioides, Epicoccum purpurascens, Glicladium roeum, Penicillium diversum* (Kremer, 1987) and *Fusarium oxysporum* (Kremer, 1987; Honda et al., 1998; Santos et al., 2011). Other authors showed that this species also produced compounds that can be of industrial use in the production of D-aminocyclases, semi-synthetic antibiotics (penicillin, cephalosporin, B-1015), hormones (lutein), bioactive peptides with immunostimulatory activities (cyclo-(l-Pro-Gly)5) and chemical pesticides (Liaw et al., 2003; Tripathi et al., 2000; Isono et al., 1993; Samanta et al., 2007; Wang et al., 2011).

Moreover, a Cyclo (L-Pro-L-Phe) isolated from the bacterium *A. faecalis* A72, showed a moderate inhibitory activity against *S. aureus* (Li et al., 2008). This same indicator strain was also effectively inhibited by the antibiotic produced by strain M3A of *A. faecalis* in Bacic and Yoch (2001) invention which supports our results. On the other hand, according to Li (2007) study, *A. faecalis* A72 showed antimicrobial activities against *B. subtilis* that corroborates with our finding. Besides, strains of *A. faecalis* type N.C.T.C. 8764 and A.T.C.C. 9220 were antagonistic against members of the same species as well as *Escherichia* (Maré and Coetzee, 1964) which is in agreement with our results. This same finding support also the results by Bacic and Yoch (2001) representing antibacterial activity of *A. faecalis* M3A, isolated from a swamp substrate, against not only all the indictor strains discussed above but also against *P. aeruginosa, M. avium* and *M. tuberculosis*.

Our data substantiates these several studies which made evident that *A. faecalis* is a potent antimicrobial agent able to inhibit bacterial species shown to be resistant to traditional antibiotic therapy. In accordance with results presented, herein, *A. faecalis* has not been previously reported to produce antibacterial compounds from tannery waste area and there is no study that elucidated its inhibitory effect against *E. chrysanthemi* used as a model strain of *E. amylovora,* the principal agent causing fire blight on pome fruits (Kotan et al., 2004).

Compounds extraction

Organic solvents have been employed to extract antibacterial, the antifungal, antimalarial and antiviral substances produced by microorganisms (Jayanth et al., 2002; Santos et al., 2011). Among the organic solvents, ethyl acetate has been widely employed (Jayanth et al., 2002; Kita et al., 1995; Li et al., 2008; Santos et al., 2011; Jebasingh and Murugan, 2011). The crude extract of the antibacterial substances prepared from *A. faecalis* was tested against *M. smegmatis, E. coli, P. aeruginosa, B. subtilis* and *S. aureus*. Thus, the antibacterial assay showed inhibition zones with different diameters (Table 3). The ethyl acetate crude extract of *E. coli* Dh5α used as control did not exhibit any inhibitory activity against the indicator strain, indicating that *A. faecalis* acts by substance(s) secreted in the medium and soluble in ethyl acetate. In contrast, the study carried out by Bacic and Yock (2001) indicated that the antibiotic produced by *A. faecalis* M3A was insoluble in organic solvents.

Determination of the kinetics

A. faecalis was oxically incubated in LB media at 30°C in a rotary shaker and the compounds production was evaluated every two hours by the well-diffusion assay.

Measurements of the optical density of the cultures during 48 h of incubation showed that there was a lag

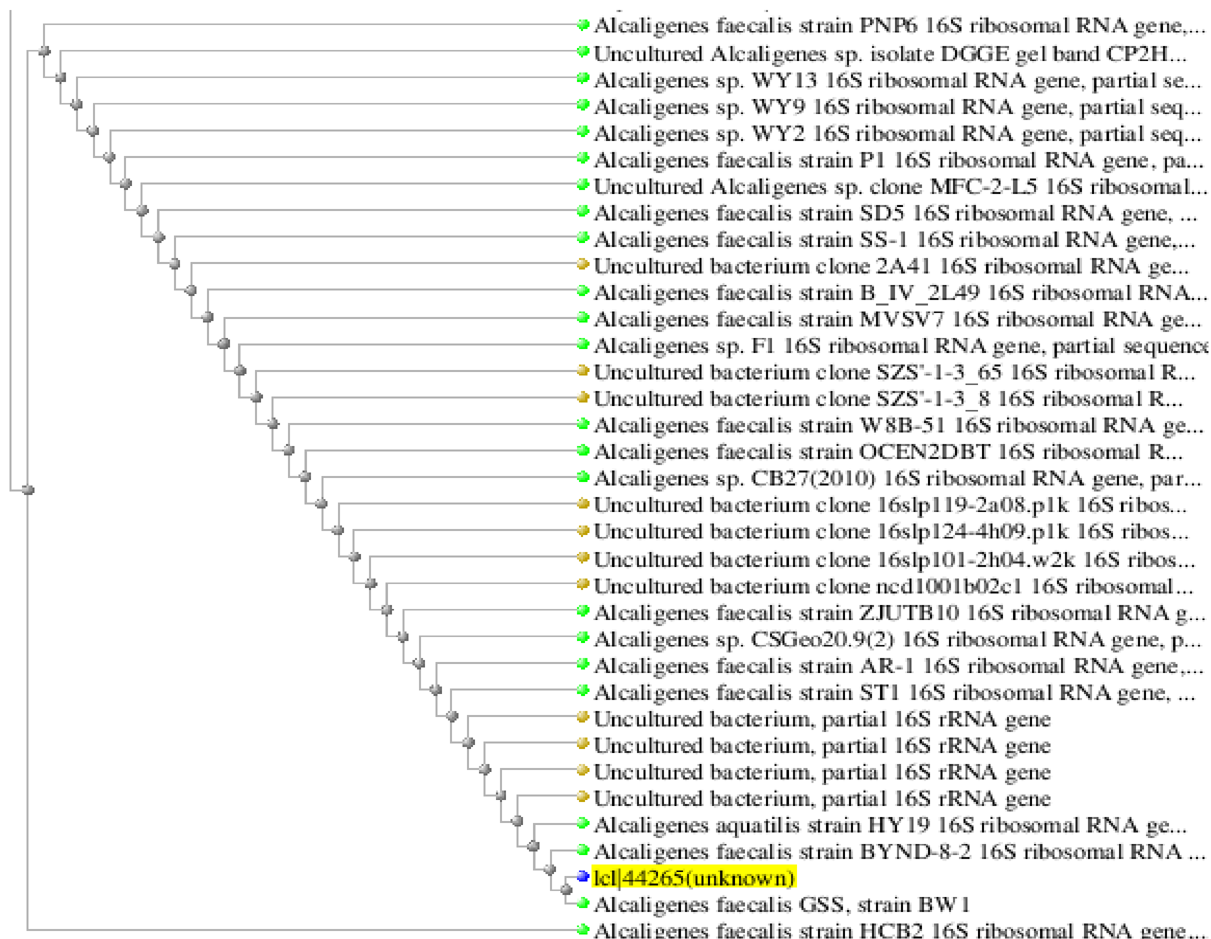

Figure 2. Phylogenetic tree showing the position of the isolate BW1. This microorganism is more closely related to *A. faecalis*. The position of BW1 is indicated by unknown in this figure.

Table 3. Diameter of inhibition halo of *A. faecalis* crude extract.

Indicator bacteria	Diameter of inhibition halo after extraction by ethyl acetate (mm)
M. smegmatis	20 ±2
E. coli	18 ±1
P. aeruginosa	16 ±0.5
B. subtilis	18 ±2
S. aureus	16 ± 1

Ethyl acetate crude extract was tested against indicator bacteria by well-diffusion agar test.

period of about 10 h before the synthesized compounds appeared in the culture medium, then, a slight inhibition of *M. smegmatis* growth occurred in the beginning of the logarithmic growth phase. An increase in the halos of inhibition diameter was observed during longer incubation period and the maximum antibacterial activity was reflected during stationary growth phase after 48 h (Figure 3). This result suggested that the growth inhibition was due to the accumulation of antimicrobial substance in the culture supernatant which was produced as a primary metabolite.

Previously, similar finding had been shown in Annamalai et al. (2011) where they demonstrated that maximum growth and chitinase production was found after 48 h of *A. faecalis* AU02 culture. Furthermore, the study conducted by Thangam and Rajkumar (2006) pointed out that the production of an extracellular protease produced by *A. faecalis* was growth-associated.

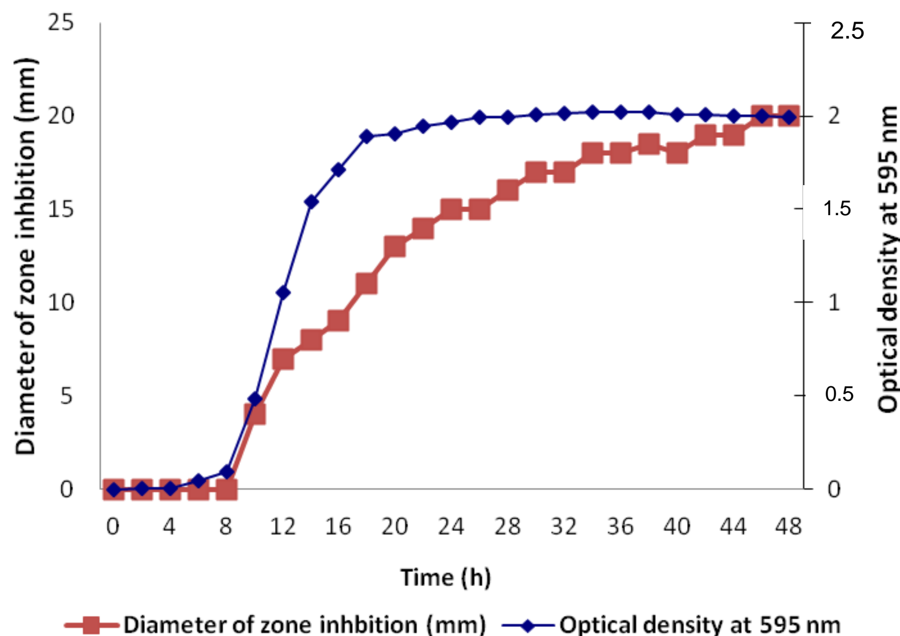

Figure 3. Growth kinetic and antibacterial agent production by *A. faecalis* (♦) growth kinetic, (■) antibacterial production. *A. faecalis* cells were cultured in LB broth and were incubated for 48 h at 37°C. The OD and antibacterial activity were measured.

Effect of heat and proteolytic enzymes

The sensitivity of the antibacterial substances produced by isolated bacterium to the heat treatment and the proteolytic enzymes proteinase K, trypsin, α-chymotrypsin and pepsin, was evaluated by measuring residual activity against *M. smegmatis* in the well-diffusion assay. *A. faecalis* compounds were not affected by all the proteases tested and their activities were also stable after heat treatments at 37, 80, 100 and 121°C which indicated non proteinaceous nature of the bioactive agent.

The storage of the bioactive substance at 4°C for six months did not influence its activity. In Bacic and Yoch (2001) investigation, the antibiotic produced by *A. faecalis* M3A was heat stable to 85°C for 10 min and its antagonistic ability was not affected neither by trypsin or proteinase K, which is compatible with our finding. However, heating either at boiling or autoclaving temperature slightly reduced the antibiotic's inhibitory action (Bacic and Yoch, 2001). In contrast, the crude extract of BW1 strain was heat stable at all tested temperatures. Thus, it is believed that the antibiotic produced by *A. faecalis* M3A is not the same bioactive substance as found in this study.

In others studies, it was noticed that the crude protease powder produced by *A. faecalis* was stable for 6 months at the storage temperature of 0-4°C which supported our finding (Thangam and Rajkumar, 2006). The antibacterial substances recovered from *A. faecalis* A72 using ethyl acetate have turned out to be L,L-Diketopiperazines (Li et al., 2008), which have been reported to possess various biological activities including antifungal (Byun et al., 2003) and antibacterial activity (Li et al., 2007). Hence, the antibacterial compounds of *A. faecalis* BW1 may be an organic compound. Further study is required to examine the extract nature of the antibacterial components.

Determination of toxicity of the extract on human erythrocytes

The toxicity of the extract of *A. faecalis* was studied using human red blood cells. At different volumes of the extract used, no hemolysis was observed thus indicating its safety for living cells (Figure 4). Unlike chloramphenicol, a synthetic antibiotic, known by his induction to lysis erythrocytes (Sharma et al., 2011), the absence of such a detrimental effect of the extract guarantees its safe therapeutic use.

To contribute efficiently to finding new drugs to fight antibiotics resistant bacteria, other investigations should be executed. Firstly, the antibacterial effect of the substances produced by the antagonistic *A. faecalis* BW1 must also be proven against *M. tuberculosis* and *E. amylovora* not only against *M. smegmatis* and *E. chrysanthemi* which are used in this study as models because they are rapid growers and are non pathogenic bacteria. Besides, preclinical study aiming at the reve-

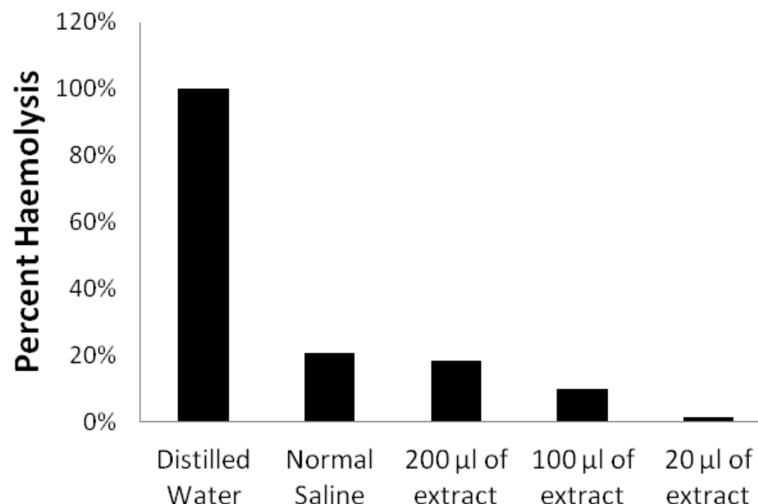

Figure 4. Cellular toxicity against human erythrocytes. Washed and diluted human erythrocytes in 0.9% NaCl solution were mixed respectively with different volumes of *A. faecalis* extract (20, 100 and 200 µl). The preparations were supplemented with 0.9% NaCl and incubated for 4 h at 37°C. The positive control contained blood treated with distilled water whereas the negative control contained blood dissolved in saline NaCl 0.9%. The OD of hemoglobin released at 540 nm was measured.

lation of the antibacterial activity of these substances *in vivo*, paired with the research of their pharmacokinetic, pharmacodynamic properties, tolerability and toxicology in different species have to be conducted. In addition, future research should be done to test the effect of this isolate under field conditions on naturally infected trees (Kotan et al., 2004). Each of these steps is important before judging whether or not it is possible to use *A. faecalis* BW1 substances as antibiotic to treat infectious diseases or as a biocontrol agent for management of fire blight.

Conclusion

The frequent emergence of resistant strains has made the whole world to be in a hurry to find new antibacterial agents with novel modes of actions (Whelana et al., 2008).

The present investigation highlights the isolation of *A. faecalis* which revealed its ability to inhibit a broad spectrum of activity against both Gram-positive and negative tested bacteria. The findings of this study suggest also that *A. faecalis* produces non proteinaceous inhibitory substances, which should be purified and identified, then determine their inhibitory minimal concentration in further work.

Finally, more studies are required to demonstrate their effectiveness *in vitro* against *M. tuberculosis* and *E. amylovora,* the real causative agents respectively of tuberculosis and fire blight, followed by other investigations with the aim of looking for the efficacy and the safety of these substances that will contribute to establish their possible beneficial effects which may suggest their probable use for therapeutic purpose against infectious diseases in mammals and plants.

ACKNOWLEDGEMENT

We thank the Regional Center of Interface, University Sidi Mohamed Ben Abdellah, Fez, Morocco for BIOSYSTEME 3130 automated gene sequencer analysis.

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