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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Full Length Research Paper

Bioformulation *Pseudomonas fluorescens* SP007s against dirty panicle disease of rice

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Two-different carrier formulations, kaolin and talc-based products were developed with *Pseudomonas fluorescens* SP007s biocontrol agent. SP007s viability in different carriers stored at room temperature (28 to 33°C) slowly declined to approximately 46.2 and 61.0% after 12-month-old shelf life. The decreased population was first found in five and month months of storage for kaolin and talc-based formulations, respectively. Field experiment with 6-foliar spray intervals (1×10^8 cfu/ml) of SP007s was conducted against naturally-occurred dirty panicle disease caused by a multiplex fungus at Suphanburi. The two bioformulations significantly reduced pathogen colonization on rice panicle and exhibited the greatest yield that correlated with increased defense-related enzyme accumulation in treated plants, compared to 4-fungicide spray intervals and nontreated control. Protection of seeds collected from colonized and noncolonized plants of dirty panicle treated with bioformulations (1×10^6 cfu/ml) and fungicides (copper hydroxide) was further determined for 12 months of storage at room temperature. The best results in reducing 6-causal fungi including *Alternaria padwickii*, *Cercospora oryzae*, *Curvularia lunata*, *Fusarium semitectum*, *Helminthosporium oryzaeqe* and *Sarocladium oryzae*; and induced seedling vigor (35%) were obtained from SP007s kaolin-based formulation evaluated at 8-month storage, but not at 12 months which indicated that these causal pathogens totally recovered their colonization except *S. oryzae*. In 8-month trials, control efficacy with dose of 1×10^6 cfu/ml SP007s seed treatment, the increase in SP007s populations relatively with the decreased colonization of pathogens could be found. SP007s in kaolin-based formulation increased GABA in SP007s treated seeds suggesting this plant bioactivator may involve plant's defense against stress conditions also.

Key words: Biocontrol, multiplex fungus causes, induced systemic resistance, protective enzymes, reduced chemical application.

INTRODUCTION

Dirty panicle of rice (*Oryza sativa* L.), one of the most important diseases in Thailand can cause great losses in

grain and seed production which makes it unacceptable for consumption and seeding. The disease caused by multiplex fungi includes *Alternaria padwickii*, *Cercospora oryzae*, *Curvularia lunata*, *Fusarium semitectum*, *Helminthosporium oryzae* and *Sarocladium oryzae*. They favor to infect plants at panicle forming stage under high temperature (28 to 33°C) and humidity (>80% RH). Some of these causal fungi also cause leaf spot or blight of rice, but the most destructiveness was found when they attack the panicle, kill the seed or exhibit seed borne infestation (Department of Agriculture, 2011) that may remain active during storage, transport and marketing. If the temperature is higher than 28°C, all causes of fungi spread rapidly from the infected to adjacent seeds. Use of contaminated seeds for planting may favor the increase in disease incidence. There are no adequate control measures to manage the disease if predisposing factors such as susceptible cultivars and weather conditions mentioned earlier are favorable for disease development. Biological control of plant pathogen is becoming an important component of plant disease management practices. This alternative control strategy can solve many persistent problems in agriculture including fungicide residues causing environmental pollution and human health hazard, and also inducing pathogen resistance (Commare et al., 2002; Cook, 2002; Bharathi et al., 2004; Chaluvaraju et al., 2004; Anitha and Rabeeth, 2009; Chen et al., 2009; Ardakani et al., 2010, 2011; Haggag and Wafaa, 2012).

The use of plant growth promoting rhizobacteria (PGPR) isolated from cauliflower root, *Pseudomonas fluorescens* SP007s as biocontrol agent in protecting various plants from several diseases caused by bacteria and fungi have been reported for multiple studies (Chuaboon et al., 2009; Prathuangwong, 2009); but not yet for dirty panicle of rice. Biocontrol mechanism by this PGPR strain SP007s revealed antibiosis; production of siderophore, auxin, and gibberellins; and inducing systemic resistance of plants (Prathuangwong et al., 2009). The phenomenon called induced systemic resistance (ISR) regulates through the activation of multiple defense compounds at sites distance from the point of pathogen attack (Prathuangwong and Buensanteai, 2007; Buensanteai et al., 2008). The inducers include pathogens, PGPR, chemicals and plant extracts (Buensanteai et al., 2009).

ISR by PGPR typically do not cause any necrotic symptoms on the host that is an activation of latent resistant mechanisms. Following applied of an inducer to plant, defense mechanisms may be triggered directly or they may be triggered only once pathogen challenges inoculations (Buensanteai et al., 2009). The defense responses activated include hypersensitive response (HR) leading to cell death and synthesis of antimicrobial compounds such as phyto-alexins and pathogenesis-related proteins (PR-proteins).

Except these defensive compounds, role in plants of GABA (γ-aminobutyric acid), a nonprotein amino acid functions in animal as a major inhibitory neurotransmitter (Erlander and Tobin, 1991) detected in nongerminated and germinated rice may also involve in plant's defense against biotic stress (Ramputh and Bown, 1996). The strains of PGPR, the major root colonizers are known to survive both in the rhizosphere, spermosphere and phyllosphere diverse that can be resident in environments (Cook, 2002). They stimulate plant growth by improving plant nutrition (Buensanteai et al., 2008), releasing plant growth regulations (Buensanteai et al., 2009), and by inhibiting plant pathogens (Van Loon et al., 1998; Chuaboon and Prathuangwong, 2007; Prathuangwong and Buensanteai, 2007; Buensanteai et al., 2009; Prathuangwong, 2009; Prathuangwong et al., 2009). These benefit bacteria can be a significant component of management practices to achieve the attainable yield.

The use of PGPR strains as biocontrol agents against dirty panicle of rice has not widely been reported although biological control of rice diseases has recently been investigated (Prathuangwong et al., 2008). The implementation of formulating biocontrol agent particular crop systems with greenhouse or field crops and seed treatment or seed coating using PGPR appears to be feasible method for dirty panicle disease (Prathuangwong et al., 2008). A formulated product must be economical to produce, easy to apply in the crop production system, efficacies with an adequate number of viable cells when used, and a shelf-stable formulated product retraining biocontrol activity comparable to fresh cells of the agent.

Delivery systems employing biocontrol agent include dust or powder, alginate pellet, and starch or extruded granule that the effective strains are necessary to be grown in various organic and inert carries, such as diatomaceous earth, manure or animal dung (Raj et al., 2003; Schisler et al., 2004; Sharathchandra et al., 2004; Amran, 2006; Pushpalatha et al., 2007; Preecha and Prathuangwong, 2009; Omer, 2010; Senthilraja et al., 2010; Siripornvisal and Trilux, 2011). Understanding of colonization ability, mechanisms of action, formulation and application should facilitate their development as reliable component in the management of sustainable agriculture system.

The objectives of this study were to evaluate: i) the efficacy of *P. fluorescens* SP007s in two formulations for control of dirty panicle, ii) viability and biocontrol efficacy after storage time, iii) spermosphere colonization of SP007s and causal pathogens, iv) formulation efficacy for control of field crop and storage seeds, and v) plant response induced by SP007s.

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Table 1. Summary of product and treatment used in the experiments.

Treatment code[1/]	Treatment detail
T1	6-foliar sprays (20 g/20 L H_20) at 20, 30, 40, 50, 60 and 70 days after planting with kaolin-based powder product (approximate 1×10^8 cfu/ml).
T2	Dose and application were same as T1, but with talc-based powder product.
T3	4-foliar sprays at 28, 42, 56, and 70 days after planting with fungicides propiconazole, copper hydroxide and difenoconazole (as conventional routine practices following label recommendation).
T4	Nontreated control.

[1/]The first spray T1 and T2 formulations with 6-month-old-shelf-life of 10^{12} and 10^9 cfu/ml was used respectively (initial 1×10^{13} cfu/ml SP007s of origin formulation).

MATERIALS AND METHODS

Formulations of *P. fluorescens* SP007s

The PGPR strain SP007s obtained from the Department of Plant Pathology, Kasetsart University, Thailand that isolated from cauliflower rhizoshere (Prathuangwong, 2009) was cultured in flasks on rotary incubator shaker at room temperature (28 to 33°C) for 48 h. Antagonistic activity of SP007s was tested against *Xanthomonas oryzae* pv. *oryzae* a cause of bacterial leaf blight of rice using a standard dual culture technique by parallel streaked the two strains onto nutrient glucose agar plates prior to further study.

To prepare biomass of SP007s cells, fermented broth was concentrated in a refrigerated and high-speed centrifuge (10,000 rpm), cells of SP007s were formulated using patent technology developed by Prathuangwong (2009) patent submission: "ISR-P", code number (0901001791) for the stabilization of *P. fluorescens* SP007s. Briefly, the inert material preparation was developed with talc; and kaolin-based powders contained talc-based: glucose : China clay : CMC: $CaCO_3$: $FeSO_4$ with 34 : 29 : 34 : 1 : 1 : 1; and kaolin : deshefix : SiO_2 : $CaCO_3$: lactose : CMC : $FeSO_4$ with 70 : 8 : 1 : 5 : 14 : 1 : 1 ratios, respectively.

The individual mixtures was moistened 5% H_2O to form workable dough and sterilized in an autoclave at 121°C for 15 min. Powder was then dried in a laminar air flow cabinet overnight prior to use in the formulation process.

Colony forming unit (cfu) of SP007s growth in sterile phosphate buffer (PBS) was determined by estimating the optical density of the bacterial suspension using spectrophotometer (CECIL 1011) adjusted to 1.2 (approximately 1×10^{13} cfu/ml) at 600 nm absorbance wavelength. The bacterial suspension was gently sprayed on talc- and kaolin-based powders at ratio 1 (biomass): 99 (other mixed ingredients aforementioned). The products were shade dried overnight to reduce the moisture content below 3% RH in a laminar flow hood.

Dried formulations were packed in sealed aluminum foiled bags (1 kg each) and stored at room temperature prior to use. To estimate number of viable cells, the standard dilution plating method with 1 to 9 aliquots of the dried powders from each formulation placed in solution of PBS plus 0.01% v/v Tension-7 and stirred for 10 min was conducted. The suspended dilutions were made and 0.2 ml aliquots plated on King's medium B. Total SP007s populations in 1 to 9 aliquots of talc and kaolin-based powders needed from mean counts of 1×10^{13} cfu/g on the day of preparing the formulations. The shelf-life of SP007s formulations stored at room temperature was determined at monthly interval throughout 12-month storage. The viable cells in each formulation were evaluated by counting cfu using standard dilution plating as aforementioned.

Effect of foliar spray with bioformulation under field experiment

Each formulation of SP007s strain was tested in farmer's field at Thongkock, Suphanburi with 3 replications of a completely randomized design arrangement. Plot size of 400×400 m^2 was maintained for all treatments. Rice seeds cv. Phitsanulok 60 were initially grown as conventional broadcast seeding with 125 kg seeds ha^{-1}. The powder product of 6-month-old shelf life (approximate 125 g ha^{-1}) mixed in water (20 g L^{-1}) for foliar spray (1×10^8 cfu/ml) until run-off using knapsack sprayer was conducted at 10-day intervals on rice plants prior to the expected infection for dirty panicle attack (the panicle formation stage) begun from 20 to 70-day-old plants (total 6-sprays). The chemical fungicides (propiconazole, difenoconazole, and copper hydroxide) at 2-week interval (begun from 28-day-old for total 4 sprays) and untreated control were maintained. No any foliar spray was done after 70-day-old plant at panicle and/or early seed formed stage. Different formulations and treatments were listed in Table 1. Panicles and/or seeds randomly selected at the panicle stage were picked intervals for evaluating natural infection by dirty panicle. Seed or grain yield was recorded at the time of harvest for all treatments. Changes of defense-related enzymes were also measured intervals at 1 day after SP007s spray and also every week after the last spray until harvest. Protein and enzyme assay following plant response investigation were later described.

Effect of seed treatment with bioformulation under storage conditions

The experiment was carried out to test the effectiveness of seed treatment with two-powder formulations of *P. fluorescens* SP007s under storage conditions of 28 to 33°C and kept in a closed plastic bag placed on a laboratory bench at Department of Plant Pathology, Kasetsart University, Bangkok. Seeds collected from healthy (symptomless) and infected panicles were washed in sterile distilled water and dressed with dried powder formulation of strain SP007s (10 month-old-shelf-life) with approximately 100 g seeds/ 0.1 g

bioformulation (approximately 1 × 10^6 cfu/ml SP007s). The entire mixture was shaker in a plastic bag for 2 to 3 min to form an even coating of the seeds, copper hydroxide and nontreated seeds were served as control treatments. All set of seeds were dried under a laminar airflow cabinet before storage in a plastic bag at room temperature. Treatments consisted of 5 replications of 1,500 seeds each. To estimate incidence of recovery and population size of SP007s and causal pathogens on storage seeds, 100 seeds were sampled monthly from each treatment. Two-separated set were evaluated with seed-washed dilutions and seeding onto solid media. Seeds were suspended in PBS buffer, sonicated, vortexed, and the dilutions were spreaded on King's medium B under amended with 150 µg/ml amplicilin and 150 µg/ml rifampicin (Nurapak and Prathuangwong, 2010); and V-8 juice agar for detection of *P. fluorescens* SP007s; and dirty panicle pathogens, respectively.

For double check, seeds were washed with PBS, subjected to slow air drying, and placed on two media above (25 seeds/plate). The plates were incubated in the growth cabinet and the cfu and colonization were recorded. In another set, treated seeds were determined for seedling vigor by agar plate method and GABA accumulation in germinated seeds was analyzed using the following procedure described:

Plant's defense response by SP007s formulations

Assay of defense-related proteins and enzymes were investigated. One gram (1 g) leaf of each treatment sampled intervals at 1 day after SP007s foliar spray and every week after the last spray until harvest was homogenized with 2 ml of 0.1 M sodium citrate buffer (pH 5.0) at 4°C. The homogenate was centrifuge for 20 min at 10000 rpm. The supernatant was used as crude enzyme extract for assaying β-1,3-glucanase (Pan et al., 1991), guaiacol peroxidase-GPX (Upadhyaya et al., 1985), peroxidase-POX (Hammerschmidt et al., 1984), phenylalanine ammonia-lyase-PAL (Prathuangwong and Buensanteai, 2007) and superoxide dismutase-SOD (Dhindsa et al., 1981). Enzyme extract was stored at -80°C until used for biochemical analysis. Protein content in the extract was determined by the method of Bradford (1976) with minor modification. Briefly, Bradford reagent was added to 0.1 ml of extract and absorbance of the mixture was read at 595 nm after a reaction time of 2 min. Sample protein content was determined from a standard curved generated with bovin serum albumin (Buensanteai et al., 2009). Analysis of GABA was extracted using the procedures described by Cohen et al. (1994), Ling et al. (1994) and Ming et al. (2011) with minor modification.

The seed-ground samples were thawed in 500 µl of a mixture of methanol: chloroform: water 12:5:3 (v/v/v). The mixture was vortexed and centrifuged at 13,000 rpm at 4°C for 15 min. The supernatant was collected, 200 µl chloroform and 400 µl water were added to the pellet. The resulting mixture was vortexed and centrifuged for 15 min at 13,000 rpm. The supernatant was collected and combined with the first supernatant and recentrifuge to collect the upper phase. The collected samples were dried in a freeze-dryer and redissolved in water. The resulting contained GABA and other amino acid. Each sample was characterized by HPLC analysis (Ming et al., 2011).

Statistical analyses

The experiments were analyzed using SPSS version 15. Data on growth, disease incidence, yield, population size of SP007s and pathogens colonized on seeds were subject to analysis of variance (ANOVA). The treatment means were compared by Duncan's multiple rage tests (Levesque, 2007).

RESULTS

Property of SP007s formulations

At each month of sampling, the average powder dissolution or viscosity and suspension of SP007s in kaolin-based formulation were significantly better than in talc-based formulation in that the kaolin-based compound tended to dissolve faster in water. No significant fitness of SP007s by these 2-formulations on rice leaves was observed at 1 day after foliar spray. However, SP007s populations were lower on the leaf surface than in the bioproducts, except for 6^{th}-month sampling date that numerous SP007s cfu were recovered. In both talc and kaolin-based products, the average culturable populations of SP007s detected from rice plants were 2-fold higher than the source bioproducts at the date of plant harvest suggesting potential adaptation and residence by SP007s (data not shown). The use of different gradients in bioproducts tested appears to determine the proportion of the total bacterial population that was culturable. Aggregation of bacterial cells could be a cause for lower culturable population. However, extensive aggregation as indicated by suspension property of powder product mentioned earlier was not evident when observed sample for active cell enumeration. The bioproduct must be quickly dissolved in water in order to activate its bacterial activity and eliciting properties that kaolin-based powder formulation dissolved in water is a faster alternative compared to talc-based.

Viability of strain SP007s cells in talc and kaolin-based formulations were estimated as a mean number of cultural colonies every month during 12 months of storage at room temperature. This biocontrol agent survived up to four and five months without any dramatic decline from the initial population (Figure 1). Although, subsequently, there was a slight decline in the population, four and five months after storage that was same 1 × 10^{12} cfu/ml obtained from both talc and kaolin based formulations. A reduction to 53.8 and 46.3% viability of SP007s was found in talc and kaolin-based formulations at 12 months of storage respectively, compared to the initial density (Figure 1).

Efficacy of *P. fluorescens* SP007s in field experiment

The *P. fluorescens* strain SP007s in both formulations kaolin and talc-based (T1 and T2) significantly reduced the percentage incidence of dirty panicle; and increased rice yield compared to fungicide spray in T3, although the last spray at 70-day-old plant with either SP007s bioproducts

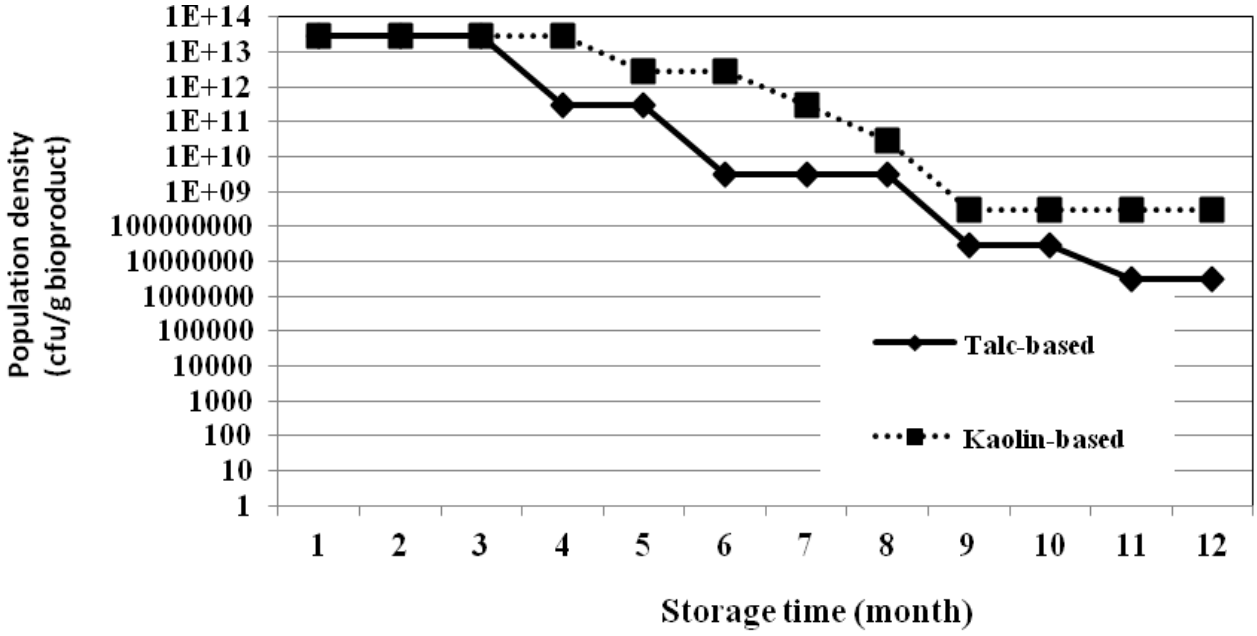

Figure 1. Survival of *Pseudomonas fluorescens* SP007s in talc and kaolin-based formulations during 12 months of storage at room temperature.

Table 2. Efficacy of *Pseudomonas fluorescens* SP007s bioformulation on disease reduction of dirty panicle and increase in yield and defense-related enzymes of rice plants under field experiment[1/].

Treatment[2/]	Disease reduction (%)[3/] Day-old-plant evaluation				Accumulation of defense related enzyme[4/]					Yield (ton/ha)
	77	84	91	98	GPX	β-1,3	PAL	POX	SOD	
T1	72.3^{b}	7.2^{b}	20.0^{b}	44.9^{a}	0.9^{a}	3.0^{a}	8.5^{a}	1.4^{a}	2.6^{b}	7.3^{a}
T2	78.7^{a}	22.9^{a}	33.3^{a}	38.0^{a}	0.9^{a}	2.6^{b}	8.0^{a}	1.0^{b}	3.0^{a}	7.3^{a}
T3	72.3^{b}	0^{c}	0^{c}	8.7^{b}	0.9^{a}	2.6^{b}	8.2^{a}	0.9^{b}	2.0^{c}	4.8^{b}
T4	0^{c}	0^{c}	0^{c}	0^{c}	0.8^{a}	2.0^{c}	5.0^{b}	0.6^{c}	1.1^{d}	2.1^{c}
CV	66.9	143.5	122.5	95.6	12.8	16.3	21.8	33.7	20.6	46.2

[1/] Means followed by same letter in a column are not significantly different according to Duncan's multiple range test (P = 0.05).
[2/] Details of treatments (T1 to T4) are same as listed in Table 1.
[3/] Treatment T1 to T3 were compared with nontreated control T4 that T3 revealed severe colonization at 84 and 91-day old plant.
[4/] Average from 10-time-evaluation, GPX = guaiacol peroxidase (unit mg^{-1} protein), β-1,3 = β-1,3-glucanase (unit mg^{-1}protein), PAL = phenylalanine ammonia-lyase (nmol tran-ciinamic acid min^{-1} mg^{-1} protein), POX = peroxidase (min^{-1} unit mg^{-1} protein) and SOD = superoxide dismutase (unit mg^{-1} protein).

or fungicides was conducted (Table 2). Overall reduction of dirty panicle by SP007s ranged between 7.2 to 78.7% depending on the different formulations used and plant ages determined that the disease symptom was firstly found 1 week after last spray (Table 2).

Fungicide treat-ment was slightly less effective in control-ling this disease that dirty panicle increased development with incidence percentages in nontreated control (data not shown). Assay of defense enzymes revealed that SP007s bioformulation induced a greater amount of enzymes in the SP007s-treated plants than the fungicide or nontreated control, although only one to two- fold increase in accumulation among GPX, β 1,3-glucnase, PAL, POX and SOD were detected in rice plant treated with *P. fluorescens* SP007s for 6-foliar spray intervals (Table 2). At plant growth stage formed for pathogen infection (70 to 98-day-old plants), no spray was carried out suggesting that SP007s mediated systemic resis-tance induction might be correlated with disease reduction.

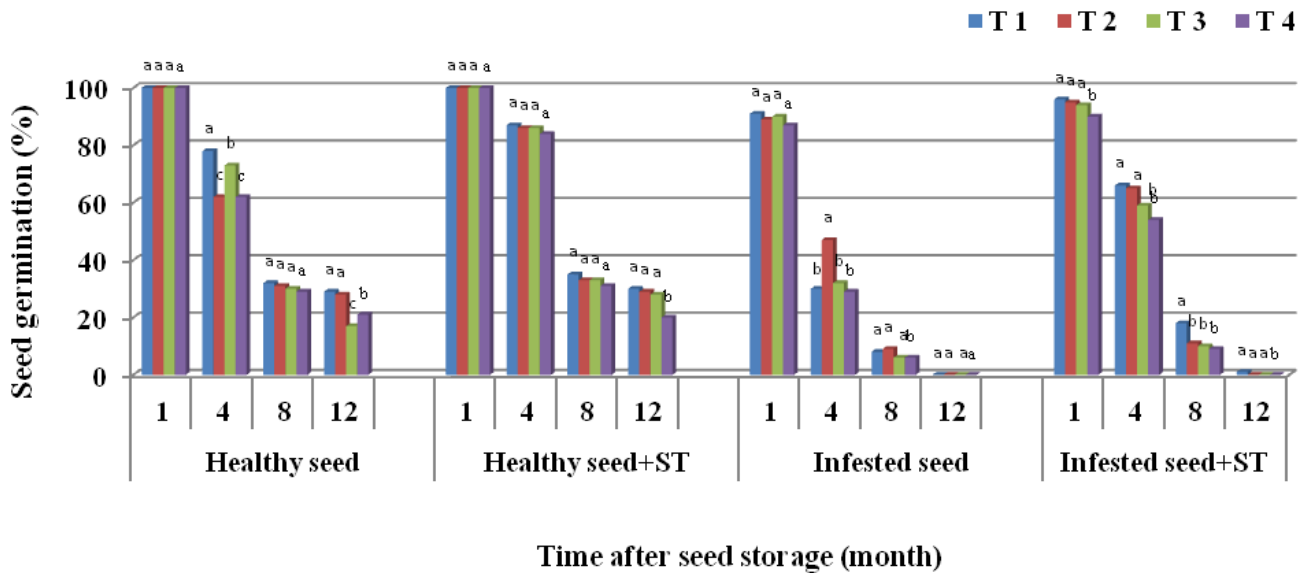

Figure 2. Seed germination of treated seeds (ST) with different products of T1 to T4 (healthy seed + ST and infested seed + ST) and nontreated control (healthy and infested seeds) evaluated monthly interval during 12-month-storage. Details of T1 to T4 are same as listed in Table 1. Letters labeling bars indicate significantly different percentage of seed germination, and treatments with the same letter are not significantly different (P = 0.05 according to Duncan's multiple rang test).

Seed treatment evaluation

The efficacy of the two formulations of *P. fluorescens* SP007s tale and kaolin-based powder products on seed germination and seedling vigor are presented in Figures 2 and 3, respectively. Percentage of seed germination obtained from treated seeds with SP007s was higher than nontreated control in either symptomless or infested seeds suggesting that *P. fluorescens* SP007s protects seeds from pathogen attack and promotes growth enhancement of rice seedlings. Noninfested or healthy and infested seeds of rice treated with 10^6 cfu/ml SP007s bioformulations (T1 and T2) showed improvement in seedling growth parameters over fungicide and untreated seeds. SP007s was found to significantly increase the vigor index of rice seedlings. The increase in main root length (9.9 cm) and shoot height (46.1 cm) including fresh (106.2 g) and dry weight (16.5 g) due to SP007s was significantly higher in T1 and T2 compared to the seedlings from T3 and T4 (Figure 3). The greatest vigor index of 43.8 was observed in the seedling treated with kaolin-based formulation containing SP007s strain (Figure 3). The positive colonization ability of SP007s as the successful colonizer of the spermosphere and its establishment on rice seeds (Figure 4) and increased seedling emergence (Figure 2) resulting in enhanced seedling vigor (Figure 3) was recorded. The relative number of 6-fungal pathogens (*A. padwickii*, *C. oryzae*, *C. lunata*, *F. semitectum*, *H. oryzae* and *S. oryzae*) in each seed treatment represented the relative population sizes of *P. fluorescens* SP007s on seeds. Quantitative differences were observed between common spermosphere and colonizer that epiphyte of SP007s was much greater than that of pathogen colonization at the 4th month of seed storage in that *S. oryzae* was completely eliminated (Figure 4). The pathogen as a colonizer was diversity greater with increase time of storage demonstrating that one time initial treatment of seeds with strain SP007s in tale-based formulation may be not sufficient to suppress these colonized pathogens throughout a longer incubation. Heavy infested seeds are also affected a success of protection with seed coating assay.

In this study, strain SP007s in kaolin-based formulation (T1) showed the best result in suppressing all 6-seedborne pathogens throughout 12-month incubation (Figure 4), resulting highestly increased seedling vigor (Figure 3) and GABA accumulation (Figure 5). Seed treatment with copper hydroxide in T3 demonstrated equivalent or less inhibition against spermosphere pathogens after 4 months but not at 8 or 12 months of storage, compared to T1 and T2 (Figure 4). However, colonization of seeds with different pathogens in nontreated control T4 was one-fold increase from the original population after 12-month storage at room temperature (Figure 4). Strain SP007s colonized and grew rapidly on treated seeds. The population levels of SP007s at 1×10^{13} cfu/g of seed within three month after treatment were obtained. This population levels decrease during the following four months of storage. No significant

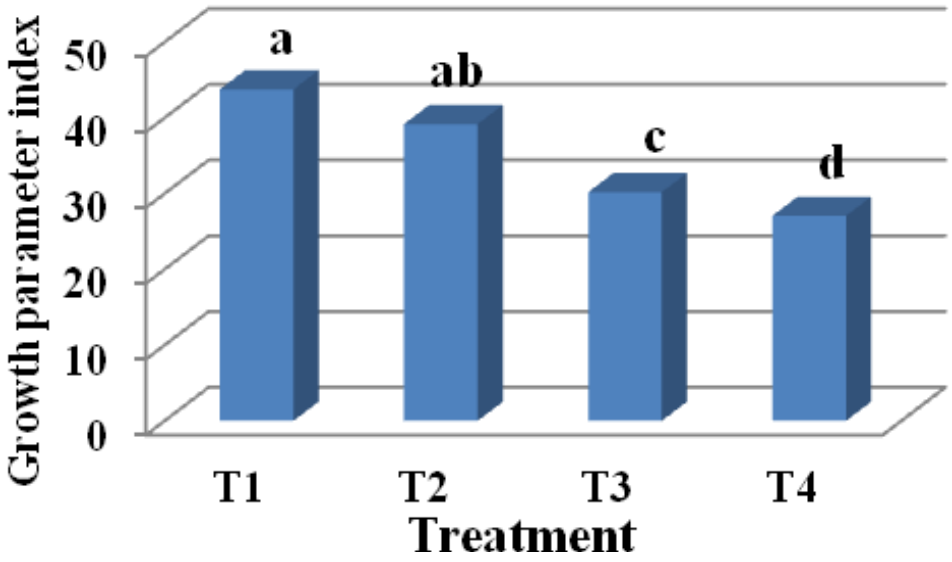

Figure 3. Effect of *Pseudomonas fluorescens* SP007s bioproducts treated infested seeds on seedling vigor of rice as shown by growth parameter index (plant growth index = root length + plant height + plant fresh weight + plant dry weight/4). Details of T1 to T4 are same as listed in Table 1. Bars with the same letters are not significantly different (P = 0.05, Duncan's multiple range test).

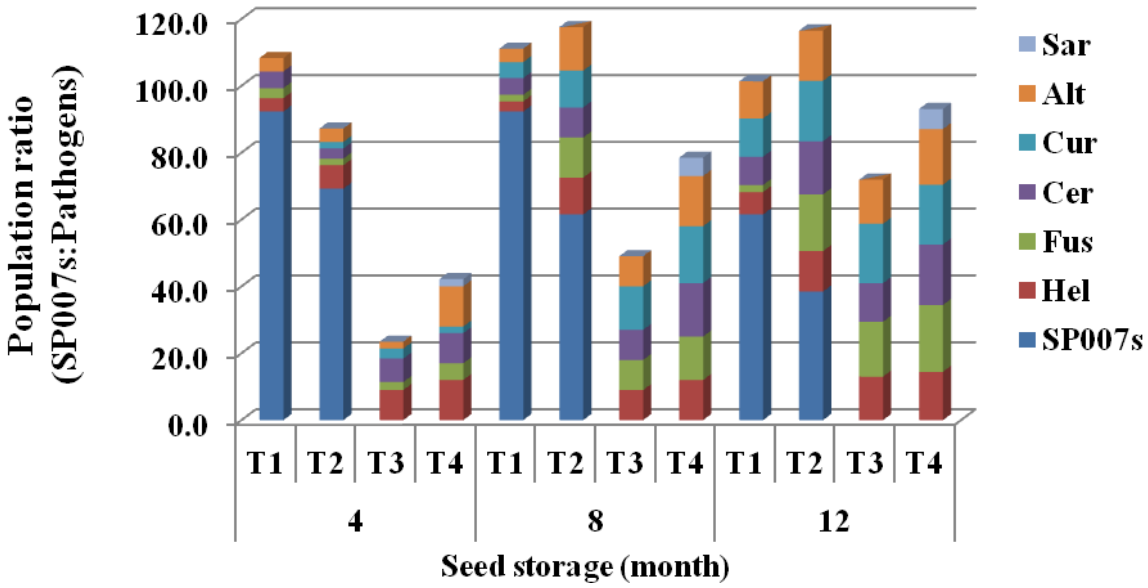

Figure 4. Effect of bioproduct seed treatment on population level of biocontrol agent *Pseudomonas fluorescens* SP007s and pathogen colonization. Hel = *Helminthosporium*, Fus = *Fusarium*, Cer = *Cercospora*, Cur = *Curvularia*, Alt = *Alternaria* and Sar = *Sarocladium*. Details of T1 to T4 are same as listed in Table 1.

differences in population levels were observed among the initial concentration inoculated (1×10^6 cfu/ml) except for T2 which attained levels of only 1×10^5 cfu/g seed after 12-month incubation. GABA was extracted from symptomless and infested seeds treated with SP007s that allowed seed germinated overnight before analysis. Change of GABA concentration in germinated seeds is shown in Figure 5. All seed treatments with T1 to T3 (SP007s

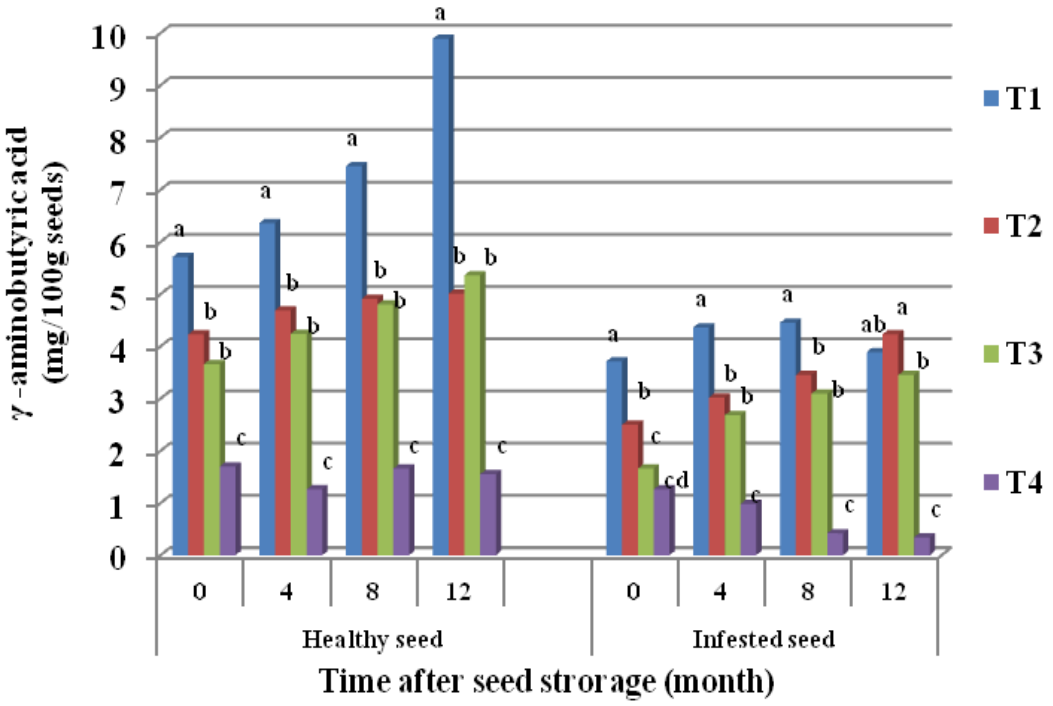

Figure 5. Increased GABA concentration in rice seeds treated with *P. fluorescens* SP007s detected during overnight germination. T1 to T4 are same as listed in Table 1. Bars with the same letters are not significantly different (P = 0.05, Duncan's multiple range test).

kaolin, SP007s talc-based formulation and copper hydroxide) significantly enhanced GABA accumulation compared to nontreated control in T4. The symptomless seeds increased higher GABA than the infestation after they were treated with SP007s. The highest GABA increase was however, found in symptomless seeds treated with SP007s kaolin-based formulation, which was 4.7 and 3.1-fold higher than nontreatment of symptomless and infected seeds respectively (Figure 5).

The data obtained clearly showed that the concentration of GABA significantly increased by rice seeds treated with *P. fluorescens* SP007s strain, and GABA concentration decreased in seeds infested with multiplex pathogens was observed. GABA was postulated to have a role in nitrogen storage and growth metabolism in plants that resulted in disease resistance by inducing PR-proteins such as β-1,3-glucanase and chitinase.

DISCUSSION

The efficacy of plant growth promoting rhizobacteria in plant pathogen inhibition, growth promotion and resistance induction in various economic crops is well understood (Commare et al., 2002; Cook, 2002; Raj et al., 2003; Bharathi et al., 2004; Chaluvaraju et al., 2004; Chuaboon and Prathuangwong, 2007; Prathuangwong and Buensanteai, 2007; Buensanteai et al., 2008; Anitha and Rabecth, 2009; Chen et al., 2009; Chuaboon et al., 2009; Ardakani et al., 2010, 2011; Haggag and Wafaa, 2012).

The results reveal in this study corroborate earlier studies and indicate a future possibility that plant growth promoting rhizobacteria bioformulations can be used to promote growth and health of economic crops (Raj et al., 2003; Chuaboon and Prathuangwong, 2007). Seed treatment and foliar application with plant growth promoting rhizobacteria bioformulations significantly en-hanced the growth of rice plants and particularly reduced the percentage of dirty particle disease incidence and severity.

Our results suggest that, the *P. fluorescens* strain SP007s in both formulations kaolin and talc-based significantly reduced the percentage incidence of dirty panicle; and increased rice yield compared to fungicide spray. In the previous study, strain SP007s survived in ISR-P® product up to 18 months of storage with 46.2% viability reduction has been reported (Chuaboon and Prathuangwong, 2007). However, with respect to the inoculum dose applied 1×10^6 and 1×10^8 cfu/ml SP007s concentrations for seed treatment and foliar spray as effective as economic threshold of SP007s utilization were recommended (Chuaboon and Prathuangwong, 2007).

Previous research showed that an 18-month-old ISR-

P® formulation with less than 53.8% of the original concentration of the biocontrol agent SP007s still provided effective control of various diseases (Prathuangwong, 2009). In this study, the loss of microbial viability during storage is one of the most important problems for microbial strains that do not form spores and formulation ingredients can improve storage survival (Siripornvisal and Trilux, 2011). In the dose of SP007s, use of kaolin allowed significantly better survival and storage stability than talc. The higher number of SP007s population in kaolin-based formulation might be due to their unique organic nature and other physio-chemical properties (Siripornvisal and Trilux, 2011). The optimal survival environment in formulation includes many variables such as temperature, moisture content, substrate (inert support and nutrients), and long term storage.

The solid substrate acts as a heterogenous source of carbon, nitrogen and minerals as well as growth factors including an ability to absorb water (Bharathi et al., 2004). Water is necessary to facilitate utilization of the nutrient substrates by the biocontrol agents. However, water excesses cause substrates to be sticky, limiting oxygen transfer and increasing the risk of saprophyte contamination; whereas, in very low moisture level, no growth of microorganism will be evident (Preecha and Prathuangwong, 2009). Although, SP007s reduced the disease incidence overall treatments, reduction of dirty panicle disease was greatest only by talc-based formulation treated plants. Similar good levels of disease control by *P. fluorescens* SP007s in field trials have been demonstrated in other economic crops (Chuaboon et al., 2009; Prathuangwong et al., 2009). These research works provided 12.4 to 49.6% control of several diseases in various crops when applied with the biocontrol agent *P. fluorescens* SP007s.

The cfu recovery of SP007s from field trial after foliar spray application demonstrated that the strain was established at stable levels in the rice phyllosphere after its initial introduction (data not shown). It could be recovered in high number (10^6 to 10^9 cfu/ml) over a period of time known to be critical for attack by dirty panicle pathogens, suggesting 6-time foliar sprays conducted in this study may be sufficient for controlling the disease.

However, application time of biocontrol agent was critical with respect to susceptible growth stage of plant for pathogen infection. The disease was severe attack at panicle stage (70 to 98-day-old plants) that no spray was recommended, frequency spray before or 1 to 2 sprays at panicle stage with SP007s bioproduct should be conducted for most effective management strategy. Recent investigation on mechanism of biocontrol by SP007s revealed that it protects plants from pathogen attack by induced different defense enzymes increased accumulation in treated plants (Prathuangwong and Buensanteai, 2007). Some of these enzymes involved in synthesis of phytoalexins and pathogenesis-related proteins that highly toxic to pathogens in different mechanisms (Cohen et al., 1994; Ramputh and Bown, 1996; Prathuangwong and Buensanteai, 2007; Buensanteai et al., 2009). Plant growth by PGPR strains include the bacterial synthesis of plant hormone indole-3-acetic acid or IAA (Buensanteai et al., 2008), cytokinin and gibberellins (Prathuangwong, 2009) that might account for plant growth promoting by PGPR-SP007s in this study.

These *Pseudomonas* bioformulations produced multiple effects, are easy to use and, most importantly, they are chemical-free (Raj et al., 2003). However, cost-effectiveness has to be worked out and if found feasible then these plant growth promoting rhizobacteria bioformulations may effectively integrate into a rice diseases control program in the near future.

Futuremore, for the future research, the effect of polymeric additives, adjuvants, surfactants on survival, stability and plant growth promoting ability of liquid bioinoculants will be performed.

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African Journal of Microbiology Research

Full Length Research Paper

Nucleotide excision repair and photoreactivation in sugarcane endophyte *Gluconacetobacter diazotrophicus* strain PAL5

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Nucleotide excision repair is a DNA repair mechanism mediated by three proteins encoded by the *uvrABC* gene homologues. This study shows that the mutant B12 of the diazotrophic sugarcane endophyte *Gluconacetobacter diazotrophicus*, carrying a transposon insertion in a *uvrC* homologue is hypersensitive to ultraviolet C irradiation. The exposure of irradiated mutant cells to visible light partially reverts the hypersensitivity, indicating that photoreactivation is another DNA repair mechanism active in *G. diazotrophicus*. Accordingly, a photolyase encoding gene is present on its genome. Therefore, this study brings experimental evidence that nucleotide excision repair and photoreactivation are DNA repair mechanisms in *G. diazotrophicus.*

Key words: Nitrogen fixing bacteria, endophytic bacteria, DNA repair, nucleotide excision repair, photolyase.

INTRODUCTION

Gluconacetobacter diazotrophicus is a nitrogen-fixing bacterium that was originally isolated as an endophyte from sugarcane (Cavalcante and Döbereiner, 1988) and from several other plant species and it has been shown to favor the growth of its host plants (Baldani and Baldani, 2005). The determination of the complete genome sequence of *G. diazotrophicus* strain PAL5 has greatly facilitated the identification of genes important in plant-bacteria interactions, including nitrogen fixation genes and genes for the production of exopolysaccharides, lipopolysaccharides, flagella and pili (Bertalan et al., 2009). It is well known that bacterial surface-exposed molecules such as lipopolysaccharides and flagellin, both from harmful and beneficial bacteria, act as microbe associated molecular patterns and may be recognized by host plants, leading to defense responses involving production of reactive oxygen species by the plant, which can kill bacteria and which are known to damage macromolecules such as nucleic acids and proteins and which may thus cause mutations (Jones and Dangl, 2006).

Tropical plants receive high levels of mutagenic ultraviolet irradiation from sunlight and thus *G. diazotrophicus*, which colonizes stems and leaves of Brazilian sugarcane plants (Cavalcante and Döbereiner, 1988), may also need to adapt to such conditions.

In a previous study, we isolated a *G. diazotrophicus* PAL5 mutant, carrying a Tn5 insertion in an *uvrC* homologous gene, and named this mutant B12 (genbank accession number EF999414) (Rouws et al., 2008). This *uvrC* homologue (genbank accession number CAP55761) is probably part of a DNA repair system of

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Abbreviations: NER, nucleotide excision repair; **UVC,** ultraviolet C.

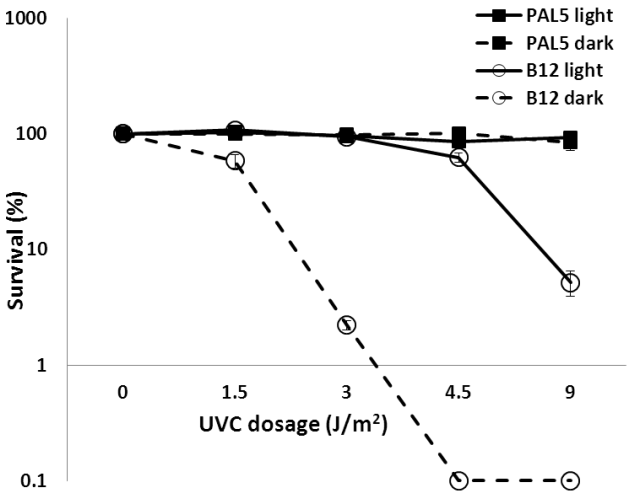

Figure 1. Survival of *G. diazotrophicus* strains after irradiation with UVC. Cells of wild-type strain PAL5 and mutant B12 were spread on agar plates and exposed to increasing dosages of UVC irradiation. Immediately after irradiation, plates were wrapped in alumium foil (dark treatment) or placed under fluorescent tube lamps for two hours (light treatment) and subsequently incubated at 30°C for three days. Colonies were then counted and the survival percentage was determined, considering the number of colonies of non-irrediated cells (dosage = 0 J/m^2) as 100%. For each treatment, the average of three indepently irradiated plates was determined and bars represent the standard error.

the Nucleotide Excision Repair (NER) pathway. NER is encoded mainly by the *uvrABC* genes, whose products act to recognize damaged nucleotides and introduce dual excisions in the DNA, after which the damaged base and some surrounding bases are removed (Kisker et al., 2013). Homologues of the *uvrAB* genes (genbank accession numbers CAP56264 and CAP56049 respectively) were also identified at different sites of the genome of *G. diazotrophicus* PAL5, so a complete UvrABC system is present in this bacterium (Bertalan et al., 2009).

The present study aimed to show functional evidence for the involvement of the PAL5 *uvrC* homolog in DNA repair, studying the effect of the mutation in mutant B12 on survival after exposure to mutagenic ultraviolet C (UVC) irradiation.

MATERIALS AND METHODS

G. diazotrophicus strain PAL5 (ATCC 49037) and mutant B12 cells were cultivated at 30°C in liquid Dygs medium (Rouws et al., 2008) to logarithmic phase (optical density of 0.6 at 600 nm), transferred to microtubes, spun down and suspended in sterile saline solution (0.7% w/v NaCl). After 10^5 times serial dilution, 50 µl aliquots of the cell suspensions, containing approximately 10^2 colony forming units, were spread on Dygs plates that were subsequently submitted to different UVC irradiation treatments.

A Mineralight UVGL-58 UVC lamp (mainly 254 nm emission) was used to irradiate plates at a distance of approximately 25 cm, resulting in an intensity of 0.5 W/m^2 (0.5 J/m^2/s), as verified using a radiometer VL-215 LM with a UVC photocell. Different UVC dosages were applied by varying the time of exposure. After exposure, the plates were immediately wrapped in aluminium foil and incubated during three days at 30°C. After incubation, bacterial colonies were counted and relative survival was determined. For each strain, the number of colony forming units of irradiated cells was divided by the number of colony forming units of non-irradiated cells (100% survival treatment) and multiplied by 100, giving the respective survival percentages. All experiments were conducted in triplicate and averages and standard errors were determined.

For phylogenetic analyses, polypeptide sequences were aligned and analyzed using the Mega 5.05 software (Tamura et al., 2011). Sequences were aligned using the integrated ClustalW option. Phylogenetic relationships were estimated using neighbor-joining (Saitou and Nei, 1987) method. Bootstrap consensus trees were inferred from 1000 replicates (Felsenstein, 1985).

RESULTS AND DISCUSSION

When the mutant B12 was exposed to increasing UVC dosages, it was hypersensitive when compared to the strain PAL5; survival of the mutant strain was reduced to below 0.1% after exposure to 6 J/m^2 (Figure 1). In contrast, survival of the strain PAL5 was not reduced even when a UVC dose of 9 J/m^2 was applied (Figure 1). Higher UVC dosages were not tested. This demonstrated that the mutation in the *uvrC* homologue strongly increased the sensitivity of *G. diazotrophicus* to mutagenic UVC irradiation, probably because of a reduced capability to repair DNA damage.

Another mechanism of DNA repair, independent of NER, is photoreactivation, which is mediated by the action of photolyases and depends on visible light as an energy-source (Essen and Klar, 2006). Therefore, it was tested if exposure of UVC irradiated bacteria to visible light had any influence on survival. For this, after irradiation, plates were placed under fluorescent tube lamps emitting white light for 2 h, then wrapped in aluminium foil and incubated during three days at 30°C. Indeed, after exposing the UVC irradiated B12 mutant to visible light, its survival increased substantially and surviving cells were present even after the highest dose of UVC (9 J/m^2) (Figure 1). In accordance with this observation, a single DNA photolyase encoding gene could be identified on the PAL5 genome (Genbank accession number YP_001601033).

Based on their substrate specificities, two classes of photolyases can be distinguished: cyclo pyrimidine dimer (CPD) photolyases and pyrimidine-pyrimidone (6-4) photolyases. Only recently the occurrence of a (6-4) photolyase has been described in prokaryotes; the bacterial species *Agorbacterium tumefaciens* was shown to carry on its genome a functional (6-4) photolyase (accession number AAK88685) along with a CPD-photolyase (accession number AAK87020) (Zhang et al., 2013). Phylogenetic analyses of the predicted aminoacid

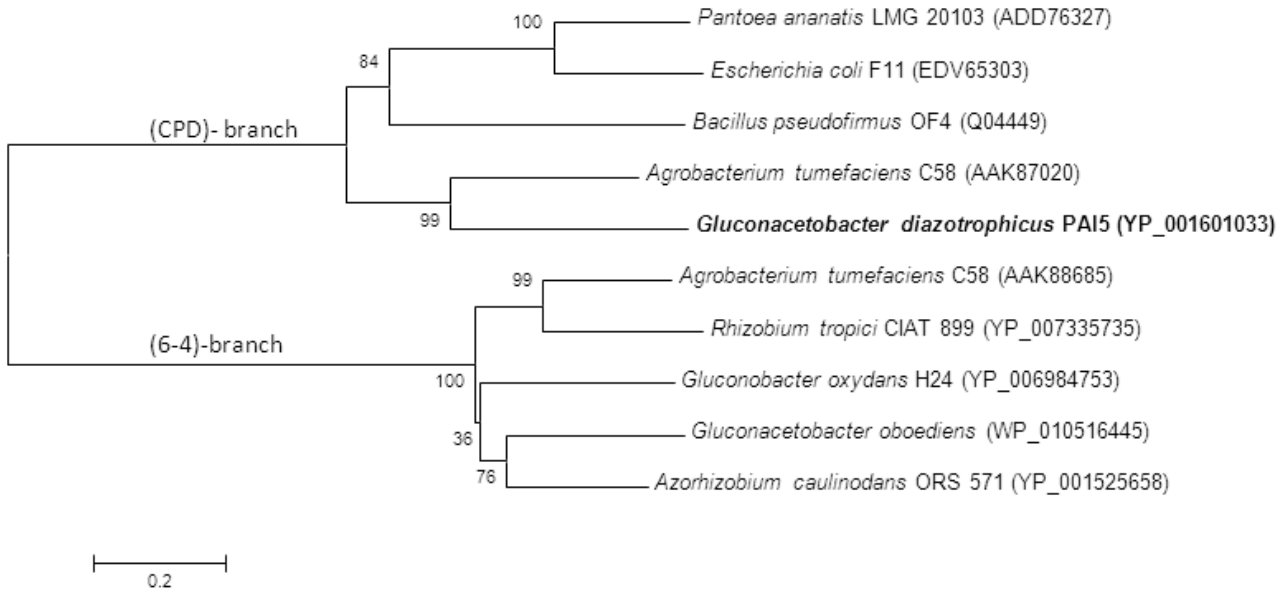

Figure 2. Phylogenetic relationship of *G. diazotrophicus* PAL5 photolyase polypeptide with other bacterial photolyase polypetides of the CPD and 6-4 photolyase classes. Bootstrap values, as % from 1000 repititions, are indicated at nodes.

sequence of the *G. diazotrophicus* photolyase (YP_001601033), conducted in the present study, showed it to pertain to the CPD class (Figure 2). Accordingly, the second His-residue of the conserved His-His-X-X-Arg motif, which is essential for the action 6-4 photolyases (Zhang et al., 2013), is absent in the predicted *G. diazotrophicus* polypeptide. Therefore, the *G. diazotrophicus* photolyase is probably of the CPD-class.

A previous study with the phyllosphere-inhabiting phytopathogen *Pseudomonas syringae* has provided evidence that NER and photoreactivation are important mechanisms in repairing DNA damage caused by solar ultraviolet B irradiation and the authors suggest that these mechanisms may play a fundamental role in enabling the bacteria to survive in association with aerial plant parts (Gunasekera and Sundin, 2006).

The results obtained in the present study represent experimental evidence that NER and photoreactivation are also active in DNA repair in *G. diazotrophicus*, a beneficial species that endophytically colonizes stems and leaves of tropical plants such as sugarcane and which may thus, depending on its localization, receive significant levels of ultraviolet B irradiation. Additional studies will be conducted to investigate the ecological relevance of these DNA repair systems during host plant colonization by this bacterial species under more natural conditions

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Full Length Research Paper

Isolation and identification of bacteria from *Xylosandrus germanus* (Blandford) (Coleoptera: Curculionidae)

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Biological control studies have been increasingly performed against agricultural and forest pests. To develop a biological control agent, bacteria was isolated from harmful pests and identified using various tests. *Xylosandrus germanus* (Blandford, 1894) (Coleoptera: Curculionidae) is a harmful pest in the hazelnut orchards and other fruit-tree cultures. In this study, we identified 16 bacteria isolates from healthy *X. germanus* collected in hazelnut orchards in Turkey. Isolates were characterized based on morphological, physiological and biochemical properties using the VITEK 2 Identification System and the fatty acid methyl esters (FAME) analysis. In addition, 16S rRNA gene sequencing of bacterial isolates was performed. Associated bacteria were identified as *Acinetobacter psychrotolerans* (2 strains), *Stenotrophomonas maltophilia*, *Pseudomonas fluorescens* (two strains), *Staphylococcus sciuri, Staphylococcus warneri*, *Pantoea agglomerans* (two strains), *Staphylococcus hominis* subsp. *hominis*, *Erwinia billingiae* (two strains), *Brevibacterium linens*, *Advenella* sp., *Pantoea cedenensis* and *Brevibacterium permense*. Several species of these bacteria are used in biological control as an antifungal and insecticidal against agricultural pest. In the future, their biological control properties will be investigated. This is the first study on the bacterial community of *X. germanus*.

Key words: *Xylosandrus germanus*, hazelnut, 16S rRNA, fatty acid methyl esters (FAME), VITEK 2, bacterial symbionts, mutualism, biological control.

INTRODUCTION

The main purpose of most agricultural studies is to increase the yield of agricultural crops. Although Turkey is first among all hazelnut producing countries (Kılıç 1994), the average yield of hazelnut per unit field is very low. Approximately 150 insect species have been detected in hazelnut orchards. However, only 10-15 of these species result in economic losses (Isık et al., 1987). Ambrosia beetles are an important pest in hazelnuts (Ak et al., 2005a, b, c).

Chemicals used against pest insects have harmful effects on the environment. Intensive use of chemicals leads to resistance in insects, and is also harmful to the environment. Biological pest control is thought to be an alternative method. Biological control provides a safety approach that is less toxic to the environment, credit to its capability of causing disease in insects, it does not harm other animals or plants. Using natural enemies against pest organism has developed the new environmentally friendly methods and microbial pest control strategies have been preferred instead of chemical pesticides worldwide.

Bacteriological studies have been made with the aim of developing biological control agents, especially against other hazelnut pest insects, such as the ambrosia beetles

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Xyleborus dispar (Sezen et al., 2007, 2008; Kati et al., 2007). Another closely related beetle, the black stem borer *Xylosandrus germanus* (Blandford 1894) (Coleoptera: Curculionidae) is also an important hazelnut pest, but its bacterial community is currently unknown. These invasive beetles are native to Asia and were first detected the US in 1932 and introduced to Europe in the 1950`s (Solomon, 1995; Lawrence, 2006). It is polyphagous and attacks a wide variety of host trees (Frank and Sadof, 2011). Bacteria are abundant and diverse on the body surface and within galleries of ambrosia and bark beetles (Hulcr et al., 2012). Here, we aimed to identify the bacterial community of *X. germanus* for the first time.

MATERIALS AND METHODS

Collection of insects and isolation of bacteria

In this study, branches with galleries creating adults of *X. germanus* in the bark were collected from the hazelnut orchards in Giresun, Turkey, in June and July 2008 and taken to the laboratory. Insects were individually put into sterilized tubes to prevent possible contamination. They were identified by Dr. Kibar Ak (Black Sea Agricultural Research Institute, Samsun, Turkey). Collected adults were surface sterilized with 70% ethanol. The adults were homogenized in a Nutrient broth (NB; containing per liter: 5 g peptone from meat; 3 g meat extract) by using a glass tissue grinder. Then, samples were ten-fold diluted. 100 µl of the suspensions were plated on a Nutrient agar (NA; containing per liter: 5 g peptone from meat; 3 g meat extract; 12 g agar-agar). Plates were incubated at 30°C for 24 or 48 h. Bacteria were selected based on their colours and colony morphologies. Then, pure cultures were prepared and these cultures were identified using various assays.

Phenotypical, physiological, biochemical properties and fatty acid methyl ester analysis of the isolated bacteria

Colony morphologies of the isolates were observed on NA by direct and stereomicroscopic observations of single colonies. Bacteria morphology and motility were examined by light microscopy of native preparations. Gram staining was performed (Claus, 1992). Endospores were observed in light microscopy using negative staining (Elcin, 1995). Temperature, NaCl and pH tolerance values were determined in NB. The VITEK 2 analysis system was used to detect biochemical properties. Fatty acid methyl ester (FAME) analysis of isolates was performed as suggested by Sasser (1990) using the Microbial Identification System (Hewlett-Packard model 5898A, Palo Alto, CA) and using the Tryptic Soy Agar (TSA) database of the Microbial Identification System software package (MIDI; Microbial ID, Inc., Newark, DE).

Molecular characterization

DNA isolation was carried out according to the procedure of Sambrook et al. (1989). The 16S rRNA gene was amplified using primers designed to anneal to conserved positions. In polymerase chain reaction (PCR), the forward primer, UNI16S-L (5'-ATTCTAGAGTTTGATCATGGCTTCA-3'), and the reverse primer, UNI16S-R (5'-ATGGTACCGTGTGACGGGCGGTGTTGTA-3') (Brosius et al., 1978) were used. The total 50 µl PCR mixture included the template DNA (10 ng), each primer (50 ng), 25 mM of each deoxyribonucleoside triphosphate (0.5 µl), 10X PCR buffer (10 µl), GoTaq polymerase (0.2 U) and distilled water.

The PCR was conducted using the following conditions: 5 min at 95°C for initial denaturation, followed by 30 amplification cycles (20 s at 95°C, 45 s at 55°C 1 min at 72°C) and 7 min at 72°C for final primer extension. All PCR products were analysed by 1.3% agarose gel electrophoresis. The resulting gene sequences (length approximately 1,400 bp) were cloned into a pGEM-T easy cloning vector. Sequencing of the cloned products was performed at Macrogen Inc. (Wageningen, Holland). These sequences comparisons were blasted against the GenBank database (Pearson, 1990; Altschul et al., 1990, 1997).

G±C analysis of Xg5 isolate

Analysis of the G±C content of the bacterial isolate Xg5 was performed using the DSMZ Identification Service. Its G±C content was determined by HPLC (Cashion et al., 1977; Tamaoka et al., 1984; Mesbah et al., 1989). The DNA was purified on hydroxyapatit according to the procedure of Cashion et al. (1977).

RESULTS

In this study, 16 bacterial isolates from *X. germanus* were identified using phenotypic, biochemical, physiological, FAME and molecular techniques. According to morphological results, five isolates were Gram-positive, the others were Gram-negative and all isolates were non-sporulating, eight isolates were motile and eight were non-motile. Moreover, the colony colours of two isolates were yellow, that of the other two isolates were orange and the others produced a creamy pigment. Four isolates had the shape of coccobacilli; five isolates were bacilli; seven isolates were cocci (Table 1).

According to pH test results, none of the isolates grow at pH 3 media; and six isolates grow at pH 5. All isolates grew at pH 7. According to heat tolerance test results, all isolates grew at 25 and 30°C, and some isolates grew at 37 and 40°C. According to NaCl tolerance test results, six isolates grow at 2% NaCl media; two isolates grow weakly; the others did not grow (Table 2). Biochemical characteristics of isolates were examined using the VITEK 2 system (Table 3 and 4). In order to identify FAME profiles of the isolates, MIS was used. In this study, according to FAME profiles, all isolates had 9-20 carbons and 46 different fatty acids were detected. Moreover, all the isolates had a C16:0 saturated fatty acid. The FAME profiles of isolates are listed in Table 5. Molecular studies of isolates were performed using 16S rRNA gene sequencing analysis. The isolates were identified as *Acinetobacter psychrotolerans* (Xg1 and Xg2), *Stenotrophomonas maltophilia* (Xg3), *Pseudomonas fluorescens* (Xg4 and Xg9), *Staphylococcus sciuri* (Xg5), *Staphylococcus warneri* (Xg6), *Pantoea agglomerans* (Xg7 and Xg15), *Staphylococcus hominis* subsp. *hominis* (Xg8), *Erwinia*

Table 1. Morphological characteristics of bacterial isolates of *Xylosandrus germanus*.

Isolate ID	Colour of colonies	Shape of colonies	Shape of bacteria	Gram stain	Motility
Xg1	Cream	Round	Coccobacili	-	-
Xg2	Cream	Round	Coccobacili	-	-
Xg3	Cream	Round	Bacili	-	+
Xg4	Cream	Wavy round	Bacili	-	+
Xg5	Cream	Round	Cocci	+	-
Xg6	Cream	Round	Cocci	+	-
Xg7	Yellow	Round	Cocci	-	+
Xg8	Cream	Round	Cocci	+	-
Xg9	Cream	Round	Bacili	-	+
Xg10	Translucent	Wavy round	Cocci	-	+
Xg11	Translucent	Round	Cocci	-	+
Xg12	Yellow-Orange	Round	Bacili	+	-
Xg13	Cream	Round	Coccobacili	-	+
Xg14	Cream	Round	Coccobacili	-	-
Xg15	Yellow	Round	Cocci	-	+
Xg16	Orange	Round	Bacili	+	-

Table 2. Physiological characteristics of bacterial isolates of *X. germanus*.

Parameter	Isolate ID															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Growth at pH 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at pH 5	-	-	+	+	-	+	+	+	+	-	-	-	-	-	-	-
Growth at pH 7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at pH 9	-	+	+	-	+	W	+	-	-	-	-	+	-	-	-	W
Growth at pH 10	-	-	+	-	+	-	W	-	-	-	-	+	-	-	-	W
Control (NB)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in NB +2% NaCl	W	-	+	-	W	+	-	-	+	+	-	+	-	+	+	+
Growth in NB +3% NaCl	-	-	-	-	W	+	-	-	+	-	-	+	-	+	-	+
Growth in NB +4% NaCl	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	+
Growth in NB +5% NaCl	-	-	-	-	-	W	-	-	+	-	-	+	-	-	-	+
Growth in NB +7% NaCl	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	+
Growth in NB +10% NaCl	-	-	-	-	-	-	-	-	+	-	-	W	-	-	-	+
Growth in NB +12% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 25˚C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 30˚C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 37°C	-	-	-	-	+	+	W	+	-	-	-	+	+	-	-	+
Growth at 40˚C	-	-	-	-	+	+	-	+	-	-	-	-	+	-	-	+

+: Growth, -: no growth, W: weak growth.

billingiae (Xg10 and Xg11), *Brevibacterium linens* (Xg12), *Advenella* sp. (Xg13), *Pantoea cedenensis* (Xg14) and *Brevibacterium permense* (Xg16) (Table 6).

DISCUSSION

In order to develop effective biological control agents, it is necessary to identify the bacterial community of insect pests. For this purpose, we aimed to identify the bacterial community of the hazelnut pest *X. germanus*. In this study, 16 bacteria isolated from *X. germanus* were identified.

According to FAME analysis and VITEK 2 results, Xg1 and Xg2 isolates were determined as *Acinetobacter*

Table 3. Biochemical characteristics of Gram negative bacterial isolates (tested with VITEK 2).

Parameter	Isolate ID										
	1	**2**	**3**	**4**	**7**	**9**	**10**	**11**	**13**	**14**	**15**
Ala-Phe-Pro-arilamidaz	-	-	+	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-
L-Pyrrlydonyl- arilamidaz	-	-	-	+	+	+	+	+	-	+	+
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-
D-Cellobiose	-	-	-	-	-	-	-	-	+	-	-
Beta-galactosidase	-	-	-	-	+	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-	-	-	-
Beta-N-acetyl-glucosaminidase	-	-	(+)	-	-	-	-	-	-	-	-
Glutamyl arilamidaz pNA	-	-	-	-	-	-	-	-	-	-	-
D-Glucose	-	-	-	-	+	+	+	+	+	+	+
Gamma-glutamyl-transferase	-	-	+	-	-	-	-	-	+	-	-
Fermentation/glucose	-	-	-	-	+	-	-	-	-	-	+
Beta-glucosidase	-	-	+	-	+	-	+	+	-	+	+
D-Maltose	-	-	-	-	-	-	-	-	-	-	-
D-Mannitol	-	-	-	-	+	-	+	+	-	+	+
D-Mannose	-	-	-	-	+	-	(-)	-	+	+	+
Beta-Xylosidase	-	-	-	-	-	-	-	-	-	-	-
Beta-Alanine arilamidaz pNA	-	-	-	-	-	-	-	-	-	-	-
L-proline arilamidaz	-	-	+	+	-	+	-	-	+	-	-
Lipase	+	-	+	-	-	-	-	-	-	-	-
Palatinose	-	-	-	-	-	-	-	-	-	-	-
Tyrosine Arilamidaz	(-)	-	-	+	-	+	+	+	-	+	-
Urease	-	-	-	-	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-	-	-	-	-
Saccharose/sucrose	-	-	-	-	+	-	-	-	-	-	+
D-Tagatose	-	-	-	-	-	-	-	-	-	-	-
D-Trehalose	-	-	-	-	+	-	+	+	-	+	+
Citrate (sodium)	+	+	+	+	-	+	-	-	+	-	-
Malonate	-	-	-	-	+	+	-	-	-	+	+
5-Keto-D-gluconate	-	-	-	-	-	-	-	-	-	-	-
L-Lactate alkalinisation	-	-	-	+	+	+	+	+	+	+	+
Alpha-Glucosidase	-	-	-	-	-	-	-	-	-	-	-
Succinate alkalinisation	-	-	+	+	+	+	+	+	+	+	-
Beta-N-Acetyl-galactosaminidase	-	-	-	-	-	-	-	-	-	-	-
Alpha-galactosidase	-	-	-	-	-	-	-	-	-	-	-
Phosphatase	-	-	+	-	+	-	+	+	-	+	(+)
Glycine arilamidaz	-	-	-	+	-	+	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-	-
Lysine decarboxylase	-	-	-	-	-	-	-	-	-	-	-
L-Histidine assimilation	+	+	-	-	-	-	-	-	-	-	-
Courmarate	-	-	-	+	-	+	-	-	-	+	-
Beta-glucoronidase	-	-	-	-	-	-	-	-	-	-	-
O/129Resistance (comp.vibrio.)	-	-	-	-	+	-	-	-	-	+	-
Glu-Gly-Arg-Arilamidaz	-	-	+	-	-	-	-	-	-	-	-
L-Malate assimilation	-	-	-	-	-	-	-	-	-	-	-
Ellman	+	-	-	-	-	-	-	-	+	-	-
L-Lactate assimilation	-	-	-	-	-	-	-	-	+	-	-

+: Growth, -: no growth, (**+**):weak growth, (**-**):almost no growth.

Table 4. Biochemical characteristics of Gram positive bacterial isolates (tested with VITEK 2).

Parameter	Isolate ID				
	5	6	8	12	16
D-Amygdalin	+	-	-	-	-
Phosphatidylinositol phospholipase C	-	-	-	-	-
D-Xylose	+	-	-	-	-
Arginine dihydrolase 1	+	+	+	+	-
Beta-galactosidase	-	-	+	-	-
Alpha-glucosidase	-	-	+	-	-
Ala-Phe-Pro arilamidaz	-	-	-	-	-
Cyclodextrin	-	-	-	-	-
L-Aspartate arilamidaz	-	-	-	-	-
Beta galactopyranosidase	-	-	-	-	-
Alpha-mannosidase	-	-	-	-	-
Phosphatase	-	-	-	-	-
Leucine arilamidaz	-	-	-	-	-
L-Proline arilamidaz	-	-	-	+	+
Beta glucuronidase	+	-	-	-	-
Alpha-galactosidase	-	-	-	-	-
L-Pyrrolydonyl-arilamidaz	-	+	-	-	-
Beta-glucuronidase	+	+	-	-	-
Alanine arilamidaz	-	-	-	+	+
Tyrosine arilamidaz	-	-	-	-	-
D-Sorbitol	-	-	-	-	-
Urease	-	+	+	-	-
Polymixin B resistance	-	-	-	-	-
D-Galactose	-	-	+	-	-
D-Ribose	+	+	-	-	-
L-Lactate alkalinization	+	-	-	+	+
Lactose	+	-	-	-	-
N-Acetyl-D-glucosamine	-	-	-	-	-
D-Maltose	+	+	+	-	-
Bacitracin resistance	+	-	-	-	-
Novobiocin resistance	+	-	-	-	-
Growth in 6.5% NaCl	+	+	+	-	-
D-Mannitol	+	-	-	-	-
D-Mannose	+	-	+	-	-
Methyl-B-D-glucopyranoside	+	-	-	-	-
Pullulan	-	-	-	-	-
D-Raffinose	-	-	-	-	-
O/129 Resistance (comp. Vibrio.)	-	+	+	-	-
Salicin	+	-	-	-	-
Saccharose/sucrose	+	+	+	-	-
D-Trehalose	+	+	+	-	-
Arginine dihydrolase 2	-	-	-	+	-
Optochin resistance	+	+	+	-	-

+: Growth, - : no growth.

haemolyticus. Jung-Sook et al. (2009) reported the presence of the following major fatty acid components in *Acinetobacter* species: 16:0, 18:1ω9c and summed feature 3. These results were consistent with ours. According to 16S rRNA gene sequencing, isolates resembled *Acinetobacter psychrotolerans* by 99%. *Acinetobacter*

Table 5. FAME profiles of bacterial isolates.

Fatty acid*	Isolate ID															
	Xg1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Saturated																
09:00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.16	-
10:00	1.57	1.67	0.33	0.13	-	-	-	-	-	-	-	-	-	-	-	-
12:00	3.27	3.77	-	2.09	-	-	3.77	-	2.52	3.89	4.14	-	3	4.18	4.08	-
14:00	0.31	0.38	1.99	0.57	0.65	-	5.37	-	0.59	5.45	6.26	-	0.81	5.75	5.52	-
15:00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16:00	19.25	20.09	6.95	36.83	2.17	1.59	30.96	0.67	36.37	33.55	35.76	0.62	29.24	37.03	30.45	0.53
17:00	1.81	1.95	0.15	0.14	-	-	0.49	-	-	-	-	-	0.75	1.08	-	-
18:00	1.95	1.77	-	1.32	0.75	8.57	0.53	5.48	1.52	-	0.55	-	0.97	0.6	-	-
19:00	-	-	-	-	-	-	-	0.99	-	-	-	-	-	-	-	-
20:00	-	-	-	-	3.98	9.61	-	9.37	-	-	-	-	-	-	-	-
Unsaturated																
15:1 iso F	-	-	1.22	-	-	-	-	-	-	-	-	-	-	-	-	-
16:1 ω9c	-	-	2.05	-	-	-	-	-	-	-	-	-	-	-	-	-
16:1 ω11c	-	-	-	-	0.36	-	-	-	-	-	-	-	-	-	-	-
17:1 ω 8c	1.46	1.9	0.44	-	-	-	-	-	-	-	-	-	-	-	-	-
17:1 iso ω10c	-	-	-	-	0.37	-	-	-	-	-	-	-	-	-	-	-
18:1 ω 9c	41.76	39.4	0.31	-	-	-	-	-	-	-	-	-	-	-	-	-
Branched																
11:0 iso	-	-	3.27	-	-	-	-	-	-	-	-	-	-	-	-	-
11:0 anteiso	-	-	0.22	-	-	-	-	-	-	-	-	-	-	-	-	-
13:0 iso	-	-	0.22	-	1.02	-	-	-	-	-	-	-	-	-	-	-
13:0 anteiso	-	-	0.21	-	-	-	-	-	-	-	-	-	-	-	-	0.32
14:0 iso	-	-	2.26	-	0.84	0.26	-	0.91	-	-	-	-	-	-	-	0.27
15:0 iso	-	-	26.58	-	42.14	2.05	-	6.39	-	-	-	4.71	-	-	-	3.63
15:0 antesio	-	-	26.24	-	22.57	50.27	-	39.56	-	-	-	54.24	-	-	-	63.97
16:0 iso	-	-	4.59	-	1.14	-	-	0.53	-	-	-	4.44	-	-	-	3.37
17:0 iso	0.35	-	2.36	-	14.31	2.71	-	4.36	-	-	-	0.84	-	-	-	0.5
17:0 antesio	-	-	0.37	-	6.96	18.06	-	7.99	-	-	-	35.14	-	-	-	27.41
18:0 iso	-	-	-	-	-	-	-	1.25	-	-	-	-	-	-	-	-
19:0 iso	-	-	-	0.35	1.49	1.53	-	10.5	0.37	-	-	-	-	0.35	-	-
19:0 antesio	-	-	-	-	1.25	5.35	-	11.35	-	-	-	-	-	-	-	-
20:0 iso	-	-	-	-	-	-	-	0.65	-	-	-	-	-	-	-	-
Hydroxy																
10:0 3OH	-	-	0.23	3.03	-	-	-	-	2.7	-	-	-	-	-	-	-
11:0 iso 3OH	-	-	1.57	-	-	-	-	-	-	-	-	-	-	-	-	-
11:0 3OH	-	-	0.15	-	-	-	-	-	-	-	-	-	-	-	-	-
12:0 2OH	4.02	3.74	-	4.7	-	-	-	-	4.17	-	-	-	-	-	-	-
12:0 iso 3OH	7.06	-	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-
12:0 3OH	-	7.14	2.05	4.35	-	-	-	-	4.41	-	-	-	-	-	-	-
13:0 2OH	-	-	1.46	-	-	-	-	-	-	-	-	-	-	-	-	-
13:0 iso 3OH	-	-	2.11	-	-	-	-	-	-	-	-	-	-	-	-	-
14:0 2OH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16:0 3OH	-	-	-	-	-	-	-	-	-	-	-	-	1.9	-	-	-
Cyc CYCLO																
17:0 cyclo	-	-	0.99	22.55	-	-	11.94	-	16.84	8.67	12.25	-	8.84	21.89	6.98	-

Table 5. Contd.

19:0 cyclo ω8c	-	-	-	2.38	-	-	0.43	-	1.49	-	-	-	1.43	4.95	-	-
Summed Feature 2 12:0 ALDE?																
16.1: iso I 14:0 3 OH	-	-	-	-	-	-	9.43	-	-	10.48	8.73	-	9.56	9.43	9.83	-
Unknown 10.928																
Summed Feature 3 16:1ω7c/16:1 ω7/6c	16.27	17.59	6.45	12.96	-	-	21.22	-	18.41	26.25	22.89	-	22.92	6.48	25.73	-
Summed Feature 8 18:1 ω7/6c	0.58	0.6	0.41	8.6	-	-	15.86	-	10.59	11.71	9.42	-	20.58	8.25	16.59	-
Summed Feature 9 17:1 iso ω9c	-	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-

*:9:0 pelargonic acid, 10:0 capric acid, 12:0 lauric acid, 14:0 myristic acid, 15:0 pentadecylic acid, 16:0 palmitic acid, 17:0 margaric acid, 18:0 stearic acid, 20:0 arachidic acid, 15:1pentadecenoic acid, 16:1 palmitoleic acid, 17:1 heptadecenoic acid, 18:1cis oleic acid.

Table 6. GenBank accession numbers of 16S rRNA genes of bacteria from *X.germanus.*

Isolate ID	Most likely identical taxonomic species	Accesion number
Xg1	*Acinetobacter psychrotolerans*	KF740570
Xg2	*Acinetobacter psychrotolerans*	KF740571
Xg3	*Stenotrophomonas maltophilia*	KF740572
Xg4	*Pseudomonas fluorescens*	KF740573
Xg5	*Staphylococcus sciuri*	KF740574
Xg6	*Staphylococcus warneri*	KF740575
Xg7	*Pantoea agglomerans*	KF740576
Xg8	*Staphylococcus hominis* subsp. *hominis*	KF740577
Xg9	*Pseudomonas fluorescens*	KF740578
Xg10	*Erwinia billingiae*	KF740579
Xg11	*Erwinia billingiae*	KF740580
Xg12	*Brevibacterium linens*	KF740581
Xg13	*Advenella* sp.	KF740582
Xg14	*Pantoea cedenensis*	KF740583
Xg15	*Pantoea agglomerans*	KF740584
Xg16	*Brevibacterium permense*	KF740585

described by Yamahira et al. (2008) had similar morphological characteristics with our Xg1 and Xg2 isolates. The genus *Acinetobacter* is widely distributed in nature; they were isolated from environmental sources such as soil, cotton, water, food and insect. In addition, *Acinetobacter* sp. were isolated from clinical specimens such as blood, feces (Brisou and Prévot, 1954; Nishimura et al., 1988; Carr et al., 2003; Baumann, 1968; Bifulco et al., 1989; Geiger et al., 2011).

Xg3 isolate was identified as *Stenotrophomonas maltophilia* according to FAME analysis, VITEK 2 and 16S rRNA sequencing. The FAME profiles are characterized by the occurrence of iso15:0, anteiso15:0, 16:1, and 16:0 as dominant components. These profiles were previously reported for *Stenotrophomonas* species (Wolf et al., 2002; Romanenko et al., 2008). *S. maltophilia* strains have been isolated from a variety of natural sources (Berg et al., 1996, 1999) and insects (Indiragandhi et al., 2007). Some members of these species are known as human pathogens (Drancourt et al., 1997; Denton and Kerr, 1998; Coenye et al., 2004). In addition, *S. maltophilia* strains are used in biological control as an antifungal agent for crops diseases (Berg et al., 1996; Jakobi et al., 1996; Minkwitz and Berg, 2001).

Xg4 and Xg9 isolates showed a low similarity with *Pseudomonas agarici* (12.9 and 35.7%, respectively) in

the FAME analyses, but closely resembled *Pseudomonas fluorescens* (99 and 95%, respectively) in the VITEK 2 analyses. Consistent with our results, Veys et al. (1989) reported the presence of three hydroxy acids (3-OH C10: 2-OH C12:0 and 3-OH C12) is characteristic of the fluorescent *Pseudomonas* species (*P. aeruginosa, P. putida and P. fluorescens*) and Camara et al. (2007) demonstrated *P. fluorescens* fatty-acid profiles contain 16:0 and 17:0 cyclo fatt acids. Xg4 and Xg9 isolates resembled *P. fluorescens* by 99%, according to 16S rRNA sequencing. Ribotyping, a method for classifying pseudomonads was used (Behrendt et al., 2003; Behrendt et al., 2007).

Based on FAME analyses, Xg5 and Xg6 isolates were identified as *Staphylococcus* sp. The Xg5 isolate was identified as *S. sciuri*, according to FAME analysis and VITEK 2 results. In previous studies, members of the genus *Staphylococcus* displayed large amounts of the fatty acids anteiso C15:0, C18:0, C20:0 and smaller but significant amounts of the fatty acids iso C15:0, C16:0, iso C17:0 ve anteiso C17:0 fatty acids (Kotilainen et al., 1990; Wieser and Busse, 2000). Our results of the 16S rRNA sequencing identified Xg5 as one of the *S. sciuri* subspecies: either *S. sciuri* subsp. *carnaticus*, *S. sciuri* subsp. *rodentium* or *S. sciuri* subsp. *sciuri* (Table 7). Thus, G±C analysis of this isolate was performed by DSMZ. We found a G±C content of 32.5% that suggested a new *S. sciuri* subspecies.

The Xg6 isolate is similar to *S. cohnii* subsp. *cohnii* based on FAME analyses. Nevertheless, according to VITEK 2 and 16S rRNA gene sequence analysis results, this isolate resembles *Staphylococcus warneri* (Table 7). Strains of *S. warneri* have been shown to grow at 40°C and are susceptiple to novobiocin (Kloos and Schleifer, 1975). These results are consistent with ours. RNA gene restriction polymorphism has been used to differentiate *S. pasteuri* from *S. wameri* (Chesneau et al., 1993). *Staphylococcus pasteuri* should be yellow in VITEK 2 tests, whereas Xg6 appeared to be creamy in our analysis. Therefore, the Xg6 isolate was identified as *S. warneri*.

Xg7 and Xg15 isolates were identified as *Pantoea agglomerans* according to VITEK 2. According to FAME analyses results, the Xg7 isolate is similar to *P. agglomerans* and the Xg15 isolate is similar to *Serratia odorifera*. 16S rRNA gene sequencing identified the Xg15 isolate as *Serratia* sp. and Xg7 as *P. agglomerans* (99%). These results were also supported by VITEK 2 analyses.

Xg8 isolate was identified as *Staphylococcus hominis* subsp. *hominis* according to FAME analysis and VITEK 2. However, 16S rRNA sequencing indicated that isolate is similar to *S. hominis* subsp. *novobiosepticus*. Kloos et al. (1998) reported *S. hominis* subsp. *novobiosepticus* is resistant to novobiocin. We found that Xg8 is susceptible to novobiocin in VITEK 2 results and therefore we concluded that Xg8 is *S. hominis* subsp. *hominis* (Table 4).

The Xg10 and Xg11 isolates were identified as *Erwinia billingiae*. The Xg10 isolate resembled *E. rhapontici* and *Sphingomonas paucimobilis,* respectively, according to FAME and VITEK 2 analyses. Geider et al. (2006) showed that C16:0 and C16:1ω7c fatty acids profiles dominated in *Erwinia* species. 16S rRNA gene sequencing has showed that this isolate is either *Erwinia billingiae* (99%) or *E. rhapontici (*98%). Mergaert et al. (1999) reported that *E. rhapontici* produces pink pigment but our Xg10 isolate produced creamy pigment. 16S rRNA gene sequencing showed that the Xg11 isolate is *E. billingiae*.

Brevibacterium sp. has higher anteiso and iso fatty acid content than other fatty acid content (Collins et al., 1983; Collins, 1992). According to FAME analysis, Xg12 and Xg16 isolates were identified as *Brevibacterim casei* and *Brevibacterium epidermidis*/*iodinum*, respectively. The major fatty acids of Brevibacterium genus have been described to be anteiso C:17 and anteiso C:I5 (Collins et al., 1980).

These isolates resemble *Dermacoccus nishinomiyaensis*. However, Stackebrandt et al. (1995) reported that anteiso-C15:0 was not found in *Dermacoccus nishinomiyaensis*. In previous studies, colony coloures of *Brevibacterium linens*, *Brevibacterium permense*, *Brevibacterium epidermidis*, *Brevibacterium iodinum* and *B. casei* were yellow-orange, orange, pale yellow, greyish and whitish grey, respectively (Bhadra et al., 2008; Gavrish et al., 2004; Collins et al., 1983). In our study, Xg12 and Xg16 isolates were yellow-orange to orange, respectively.

16S rRNA sequencing showed that the isolates belong to the Brevibacteria. Morhopological studies showed that Xg12 and Xg16 isolates are *B. linens*, *B. permense*, respectively. *Brevibacterium* species have been isolated from insect (Katı et al., 2010).

The Xg13 isolate was highly similar to *Advenella kashmirensis* and *Advenella incenata* (98%) using 16S rRNA sequencing. 16:0 and 18:1ω7c fatty acids dominate in *Advenalla* sp. (Coenye et al., 2005). This is in accordance with our study.

The Xg14 isolate resembles *Pseudomonas luteola* (95%) according to FAME analysis and VITEK 2 results. It resembles *Pantoae cedenensis* (99%) according 16S rRNA sequencing. Fatty acids contents of this isolate were very similar to Mergaert et al. (1993). *Pseudomonas luteola* is yellow pigment (Holmes et al., 1987), but *Pantoae cedenensis* is creamy (Sezen et al., 2008), like Xg4 in our study.

As a result, bacteria isolated from *X. germanus* were identified in this study. In future, biological control properties of these bacteria will be investigated. In previous studies, several species of *Acinetobacter*, *Stenotrophomonas*, *Pantoea*, *Brevibacterium* and *Pseudomonas* bacteria identified in this study exhibited antifungal or insecticidal activities (Selvakumara et al., 2011; Trotel-Aziz et al., 2008; Jankiewicz et al., 2012).

Table 7. Identity of isolates according to VITEK 2, FAME profiles and 16S rRNA sequencing.

Isolate ID	FAME profile	Similarity (%)	VITEK 2 analysis	Similarity (%)	16S rRNA results	Closest match GenBank accession no.	Similarity (%)
Xg1	*Acinetobacter haemolyticus*	84.6	*Acinetobacter haemolyticus*	91	*Acinetobacter psychrotolerans*	AB207814	99
Xg2	*Acinetobacter haemolyticus*	76.5	*Acinetobacter haemolyticus*	91	*Acinetobacter psychrotolerans*	AB207814	99
Xg3	*Stenotrophomonas maltophilia*	51.1	*Stenotrophomonas maltophilia*	99	*Stenotrophomonas maltophilia* strain ISSDS-429	EF620448	99
Xg4	*Pseudomonas agarici*	12.9	*Pseudomonas fluorescens*	99	*Pseudomonas fluorescens* strain ESR94	EF602564	99
	Staphylococcus schleiferi	54.3	*Staphylococcus sciuri*	97	*Staphylococcus sciuri* subsp. *carnaticus*	AB233331	99
Xg5	*Staphylococcus sciuri*	43.3			*Staphylococcus sciuri* subsp. *rodentium*	AB233332	99
					Staphylococcus sciuri subsp. *sciuri* strain DSM 20345	NR_025520	99
Xg6	*Staphylococcus cohnii* subsp. *cohnii*	23.8	*Staphylococcus warneri*	99	*Staphylococcus warneri* strain E21	GU397393	99
Xg7	*Raoultella terrigena*	76.2	*Pantoea agglomerans*	98	*Staphylococcus pasteuri* strain SSL11	EU373323	99
					Pantoea agglomerans strain PGHL1	EF050808	99
	Pantoea agglomerans GC subgroup B (Enterobacter)	75.7			*Pantoea ananatis* strain SAD2-6	HQ236020	99
Xg8	*Staphylococcus hominis* subsp. *hominis*	66.6	*Staphylococcus hominis* subsp. *hominis*	94	*Staphylococcus hominis* subsp. *novobiosepticus* strain: GTC 1228	AB233326	99
Xg9	*Pseudomonas agarici*	35.7	*Pseudomonas fluorescens*	95	*Pseudomonas fluorescens* strain CN078	EU364534	99
Xg10	*Erwinia rhapontici*	71.2	*Sphingomonas paucimobilis*	89	*Erwinia billingiae* strain Eb661	AM055711	99
					Erwinia rhapontici strain M52	HM008951	98
					Erwinia persicinus strain 52	AM184098	98
Xg11	*Erwinia amylovora*	57.7	*Sphingomonas paucimobilis*	89	*Erwinia billingiae* strain Eb661	FP236843	99

Table 7. Contd.

Xg12	*Brevibacterium casei*	80.5	*Dermacoccus nishinomiyaensis*/*Kytococcus sedentarius*	93	*Brevibacterium aureum* strain Enb17	AY299093	99
					Brevibacterium linens strain VKM Ac-2119	AY243345	99
					Brevibacterium iodinum strain ATCC 15728	FJ652620	98
					Brevibacterium epidermidis strain ZJB-07021	EU046495	98
					Brevibacterium permense strain VKM Ac-2280	NR_025732	98
Xg13	*Pantoea agglomerans* GC subgroup C (Enterobacter)	61.7	*Acinetobacter lwoffi*	93	*Advenella kashmirensis* strain 445A	AJ864471	98
					Advenella incenata	AM944735	98
Xg14	*Ewingella americana*	76.5	*Pseudomonas luteola*	95	*Pantoea cedenensis* strain 16-CDF	FJ811867	99
Xg15	*Serratia odorifera*	75.9	*Pantoea agglomerans*	95	*Pantoea agglomerans* strain EQH21	FJ999950	99
					Pantoea ananatis strain SAD2-6	HQ236020	98
Xg16	*Brevibacterium epidermidis*/*iodinum*	81.6	*Dermacoccus nishinomiyaensis*/ *Kytococcus sedentarius*	97	*Brevibacterium epidermidis* strain SW34	GU576981	99
					Brevibacterium linens	AB211980	98
					Brevibacterium aureum strain Enb15	AY299092	99
					Brevibacterium iodinum strain DSM 2062	NR_026241	98
					Brevibacterium permense strain VKM Ac 2280	NR_025732	98

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Full Length Research Paper

The effect of hydroxycinnamic acids on growth and H^+-ATPase activity of the wine spoilage yeast, *Dekkera bruxellensis*

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Hydroxycinnamic acids are lipophilic compounds naturally present in grape must, and proposed to have antimicrobial properties. Consequently, microorganisms that grow in media containing these acids must have efficient adaptation mechanisms. In *Saccharomyces cerevisiae* hydroxycinnamic acids enter into the cell where they are deprotonated causing a decrease in internal pH, this variation in the intracellular pH is counteracted by an increase in the activity of the H^+- ATPase pump Pma1p. *Dekkera bruxellensis* however, is able to transform hydroxycinnamic acids into volatile-less toxic derivates, a mechanism used by few yeast species. Nonetheless, *D. bruxellensis* could also have an adaptation mechanism similar to that of *S. cerevisiae.* Our results showed that hydroxycinnamic acids caused a longer *lag* phase during *D. bruxellensis* growth, particularly when supplementing media with ferulic acid. Additionally, extracellular pH decreased while Pma1p activity increased during *lag* phase in media supplemented with *p*-coumaric acid. These results suggest the existence of a complementary mechanism of resistance to hydroxycinammic acids in *D. bruxellensis* which involves the H^+- ATPase pump Pma1p.

Key words: *Dekkera bruxellensis*, H^+-ATPase Pma1p, *p*-coumaric acid.

INTRODUCTION

Hydroxycinnamic acids (HCAs) are the most important group of polyphenols present in wine. These compounds which are initially esterified with tartaric acid are released into the grape juice by the action of cinnamoyl esterase enzymes naturally found in the grape must. Within hydroxycinnamic acids, the most important compounds are cafeic, *p*-coumaric and ferulic acid (Vrhovšek, 1998). These molecules are weak acids with a lipophilic character and show antioxidant and antimicrobial properties. In addition, these compounds are precursors of volatile phenols (4-ethylphenol, 4-ethylguaiacol, 4-vinylphenol, 4-vinylguaiacol) that impact negatively on wine sensory properties.

Since wine pH hydroxycinnamic acids are protonated, they can freely diffuse into the cell where they release protons affecting the cellular capacity to maintain pH homeostasis, blocking the transport of substrates, and finally inhibiting growth (Piper et al., 2001; Beales, 2004). Some studies have reported a H^+-ATPase enzyme (Pma1p) in *S. cerevisiae* which pumps protons to the extracellular media in response to increased concentration of weak acid in the culture media (Chambel et

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al., 1999; Viegas et al., 1998). Thus, Pma1p constitutes a support mechanism to counteract the decrease in internal pH caused by the presence of weak acids (Sá-Correia et al., 1989; Viegas and Sá-Correia, 1995; Viegas et al., 1995; Carmelo et al., 1997). Indeed, octanoic acid and cinnamic acid increase the activity of Pma1p in *S. cerevisiae* and extend the duration of the *lag* phase (Viegas et al., 1998; Chambel et al., 1999). During this extended period cells adapt to the toxic effects of weak acids, and the duration of *lag* phase would depend on the concentration of these acids.

Thus, for *S. cerevisiae* there is a direct co-relation between the antimicrobial effects of weak acids, the duration of *lag* phase and the activity of H^+-ATPase pump Pma1p. *D. bruxellensis* responds to hydroxycinnamic acids toxicity by metabolizing these compounds into less toxic volatile metabolites (Dias et al., 2003). Nevertheless, we have observed that *p*-coumaric acid affects *D. bruxellensis* growth extending *lag* phase duration. Similarly to described Curtin et al. (2012) and Piskur et al. (2012), we found that *D. bruxellensis* L1359 has a Pma1p protein, which is involved in a mechanism of adaptation to hydroxycinammic acids, and seems to be activated during *lag* phase in the presence of these acids.

MATERIALS AND METHODS

Strains and culture conditions

D. bruxellensis L1359 was obtained from the strain collection of the Applied Microbiology and Biotechnology Laboratory of the Universidad de Santiago de Chile.

D. bruxellensis growth was evaluated in microtiter plates sealed with gas permeable membranes. Briefly, colonies from YPD agar were inoculated into YPD media (0.5% peptone, 0.5% yeast extract, 4% glucose, pH 6.0) and grown overnight at 28°C with shaking (150 rpm). This culture was then inoculated in 200 µL of Synthetic Dextrose Minimal Medium (SD) (glucose 20 g/L, YNB 6.7 g/L, pH 4.3 (unbuffered)) containing different concentrations of hydroxycinnamic acids at a cell density of 1 x 10^6 cells/ml. SD media contained 0, 25, 50, 75 and 100 mg/L of *p*-coumaric acid, caffeic acid and ferulic acid (Sigma-Aldrich, USA), each condition was replicated three times. The pH value of the media was not modified by the presence of HCA. Plates were maintained at 28°C for three days, with 10 s of agitation (500 rpm) every hour. Growth was monitored by measuring optical density at 600 nm using Elx 808 multiplates reader (BioTek, USA) coupled to the Gen5 program (BioTeK, USA).

The specific growth rate (□) was estimated from the slope of the growth curve during exponential phase according to the equation $x_t = x_0+\mu t$, where: x_t and x_0 correspond to the biomass concentration or the optical density (OD) at time t (h) and t = 0, respectively (Barata et al., 2008). The R^2 values of the curves were 0.996 or higher in all cases.

Lag phase duration was determined mathematically according to Buchanan and Cygnarowicz (1990) as the time when the second derivative of the logarithm of the growth curve reaches a maximum value.

Evaluation of extracellular acidification during yeast growth

Extracellular acidification was evaluated as described by Chambel et al. (1999). Yeast colonies were grown overnight in YPD media at 28°C with shaking (150 rpm). Cultures were then inoculated in 1 L of SD media (control) or SD media supplemented with 100 mg/L of *p*-coumaric acid. SD media were inoculated at 5x10^5 cell/ml and incubated at 28°C with shaking (150 rpm). All cultures were performed in triplicate. Cell counts were determined using a Neubauer chamber as described previously (Becker et al., 1999). Samples (5 ml) were taken periodically during yeast growth, centrifuged at 3000 x*g* for 5 min and pH was determined from the supernatant using a pH meter (HI 2221 Calibration Check Ph/ORP Meter, Hanna Instruments, USA).

Determination of acetic acid during growth by HPLC

The following acetic acid was done using the technique of high performance liquid chromatography (HPLC) (Shimadzu Scientific Instruments, Colombia, MD, USA). The ion exchange column Bio-Rad HPX-87H was used, a mobile phase of sulfuric acid (5 mmol/L), at a flow rate of 0.4 mL/ min, IR and UV detector at 200 nm, at 55°C (Ross et al., 2009). Detection limit was 0.05 g/L.

Plasma membrane ATPase Pma1p activity assay

The activity of the plasma membrane ATPase Pma1p was estimated from the rate of phosphate production after ATP hydrolysis (Baykov et al., 1988). First, cell cultures were grown in SD media (supplemented and unsupplemented with *p*-coumaric acid) as described above. Culture samples (100 ml) were taken during *lag* phase (12 h for unsupplemented and 48 h for supplemented), exponential phase (48 h for unsupplemented and 144 h for supplemented) and stationary phase (168 h for unsupplemented and 216 h for supplemented) and centrifuged at 3000 x*g* for 2 min at room temperature. After centrifugation, cell pellets were resuspended in 800 µl of SD medium for 5 min at room temperature with occasional agitation. Cells were then disrupted with glass beads (0.5 mm; Sigma, St. Louis, USA) to obtain crude membrane suspensions as previously described by Serrano (1983).

To avoid interfering ATP hydrolysing or phosphatase activities specific inhibitors were used for the enzymatic assay. Thus, plasma membrane ATPase activity was assayed in crude membrane suspensions using 50 mmol/L of buffer MES (2-(*N*-morpholino) ethanesulfonic acid) pH 5.7, 10 mmol/L MgSO4, 50 mmol/L KCl, 0.2 mmol/L ammonium heptamolybdate (phosphatase inhibitor), 5 mmol/L NaN_3 (ATPase mitochondrial inhibitor), 100 mmol/L KNO_3 (vacuolar ATPase inhibitor) and 2 mmol/L ATP (Sigma, St. Louis, USA). Under these conditions, ATPase activity could be attributed predominantly to plasma membrane H^+-ATPase Pma1p. Phosphate released by Pma1p activity was then quantified according to Baykov et al. (1988). Phosphate forms a bright green complex with malachite green in acid conditions which can be followed spectrophotometrically at 630 nm. Plasma membrane ATPase specific activity (U/mg) was calculated from the rate of phosphate production and was expressed as micromoles of phosphate released per min (U) per mg of protein. Protein concentration in crude membrane suspensions was determined according to Bradford (1976) using bovine serum albumin as standard.

Statistical analysis of the data

Statistical comparisons were made using the Student's t-test or analysis of variance (ANOVA) as indicated and considered significant differences at $p \leq 0.05$. This analysis was carried out using Statgraphics Plus, version 5.1 (StatPoint Technologies, Warrenton, Virginia, USA).

Table 1. Specific growth rate (μ *10^{-3}(h^{-1})) of the strain *D. bruxellensis* L1359 grown in SD media containing different concentrations of hydroxycinnamic acids.

Medium/HCA (mg/L)	0	25	50	75	100
p-Coumaric acid	32 ± 0.46	33 ± 0.25	34 ± 0.21	28 ± 0.25	27 ± 0.31
Ferulic acid	32 ± 0.46	37 ± 0.25	30 ± 1.53	27 ± 0.06	26 ± 0.21
Caffeic acid	32 ± 0.46	33 ± 0.15	32 ± 0.12	28 ± 0.25	27 ± 0.15

All results were expressed as means of three replicates.

Table 2. *Lag* phase duration (h) of the strain *D. bruxellensis* L1359 grown in SD media containing different concentrations of HCA.

HCA (mg/L)	*lag* (h)				
	0	25	50	75	100
p-Coumaric acid	2.0 ± 0.0	3.0± 0.0	3.5 ± 0.7	7.5 ± 0.7	15.0 ±0.0
Ferulic acid	2.0 ±0.0	3.5 ± 0.7	4.0±0.0	8.5 ± 0.7	16.5 ± 0.7
Caffeic acid	2.0±0.0	3.0± 0.0	3.0± 0.0	5.5 ± 0.7	12 ± 0.7

All results are expressed as means of three replicates.

RESULTS AND DISCUSSION

Inhibition of yeast growth by hydroxycinnamic acids

The effect of hydroxycinnamic acids on *D. bruxellensis* growth depends on their concentration (Baranowski et al., 1980). In this work, we assessed the effect of several concentrations (0-100 mg/L) of *p*-coumaric acid, caffeic acid and ferulic acid on the cell growth of *D. bruxellensis* L-1359. At concentrations of 25 mg/L of HCA there was a small but significative increase on growth rate as compared to the control (Table 1). In contrast, concentrations of 75 mg/L or more affected negatively *D. bruxellensis* growth rate. This inhibition of cell growth can be expected since HCAs act as antimicrobial agents (Baranowski et al., 1980). Similar results have been reported previously showing that ferulic acid at 388 mg/L inhibited the growth of several *D. bruxellensis* strains (Harris et al., 2008). Positive effects of HCAs on the growth rate of different isolates of *Dekkera/Brettanomyces* spp. have also been reported (Godoy et al*.,* 2009). These findings suggest that the effect of HCAs on cell growth might be strain-dependent.

Ferulic acid showed the most negative effect on growth rate (Table 1). Baranowski et al*.* (1980) reported that the inhibitory capacity of HCAs is proportionally inverse to its polarity, making ferulic acid the most inhibitory of the acids assayed in this study.

The addition of HCAs also altered the length of the *lag* phase for *D. bruxellensis* (Table 2). At 100 mg/L *p*-coumaric acid extended the duration of the *lag* phase from 2 (control) to 15 h (Table 2), while ferulic acid and caffeic acid increased *lag* phase to 16.5 and 12 h, respectively. Similar results showing a longer *lag* phase have been reported for *S. cerevisiae* growing in media supplemented with *p*-coumaric acid (Baranowski et al., 1980) and for *D. bruxellensis* exposed to p-coumaric acid (Dias et al., 2003) and ferulic acid (Harris et al., 2008, 2010).

Evaluation of extracellular acidification during yeast growth

Weak acids can enter the cell undissociated and once inside dissociate affecting intracellular pH and potentially cell metabolism. Thus, to maintain intracellular homeostasis the yeast cell requires mechanisms that can reduce the concentration of protons in the cytoplasm. It has been reported that *S. cerevisiae* can decrease extracellular pH during the first hours of cell growth (*lag* phase) when exposed to weak acids such as cinnamic acid (Chambel et al. 1999) and sorbic and acetic acids (Stratford et al., 2013). These results suggest that the proton pump Pma1p is stimulated when *S. cerevisiae* is cultured in the presence of weak acids.

Similarly, in this study extracellular pH decreased during *lag* phase when *D. bruxellensis* was grown in media containing 100 mg/L of *p*-coumaric acid (Figure 1). Although a similar decrease was initially observed for the control, extracellular pH then increased to pH 4.3 and remained stable up to 100 h to decrease again to pH 4.1 (Figure 1). In media containing *p*-coumaric acid extracellular pH decreased steadily to pH 3.8 during *lag* phase (96 h). Subsequently, extracellular pH increased to similar values than the control (pH 4.1). Sigler and Hofer (1991) suggested that while the production of organic acids during yeast growth contributes to extracellular acidification, this only occurs during exponential phase. In this work, according to HPLC data, *D. bruxellensis*

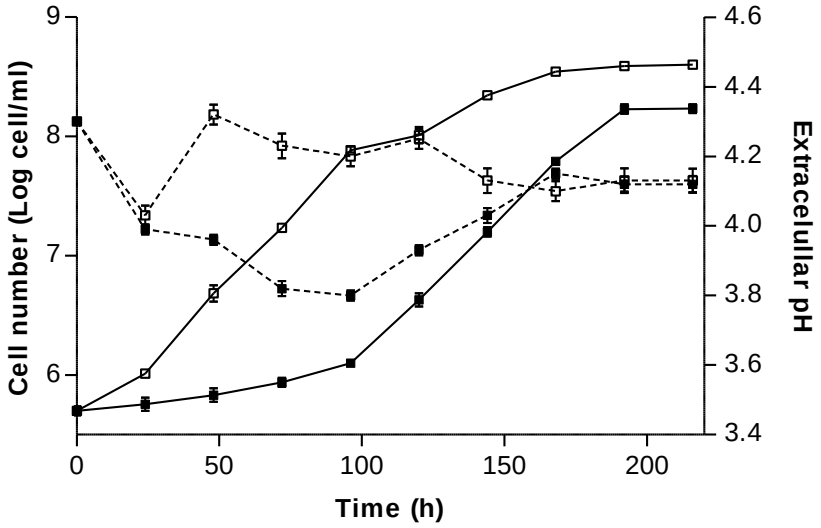

Figure 1. Cell number (solid line) and extracellular pH (dotted line) during growth of *D. bruxellensis* L1359 in SD media (open squares) and in SD media containing *p*-coumaric acid at 100 mg/L (filled squares). All the experiments were conducted in triplicate.

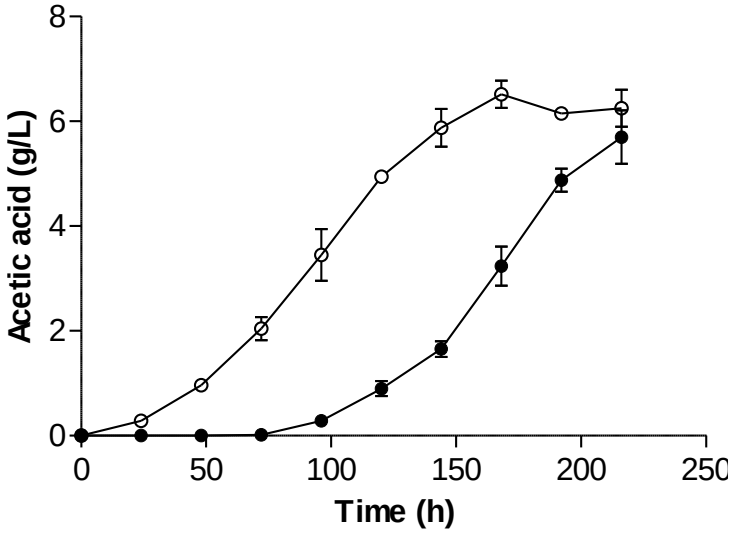

Figure 2. Acetic acid production during growth curve of *D. bruxellensis* L1359 in SD media (open circles) and in SD media containing *p*-coumaric acid at 100 mg/L (filled circle). All experiments were conducted in duplicate.

produced acetic acid during exponential phase (Figure 2) which is in agreement with literature (Geros et al., 2000; Leite et al., 2012), and in *lag* phase, acetic acid production was not detected.

These findings suggest that the pH decrease observed during *lag* phase in media supplemented with *p*-coumaric acid might be the result of H^+- ATPase activity, similar to what has been observed in *S. cerevisiae* (Chambel et al., 1999).

Quantification of Pma1p activity in the presence of p-coumaric acid

Pma1p activity was quantified on membrane protein extracts from cultures grown in SD media or SD media containing *p*-coumaric acid. Since Pma1p activity was estimated from the rate of phosphate production after ATP hydrolysis, different compounds were used to inhibit other enzymes capable of hydrolyzing ATP or molecules

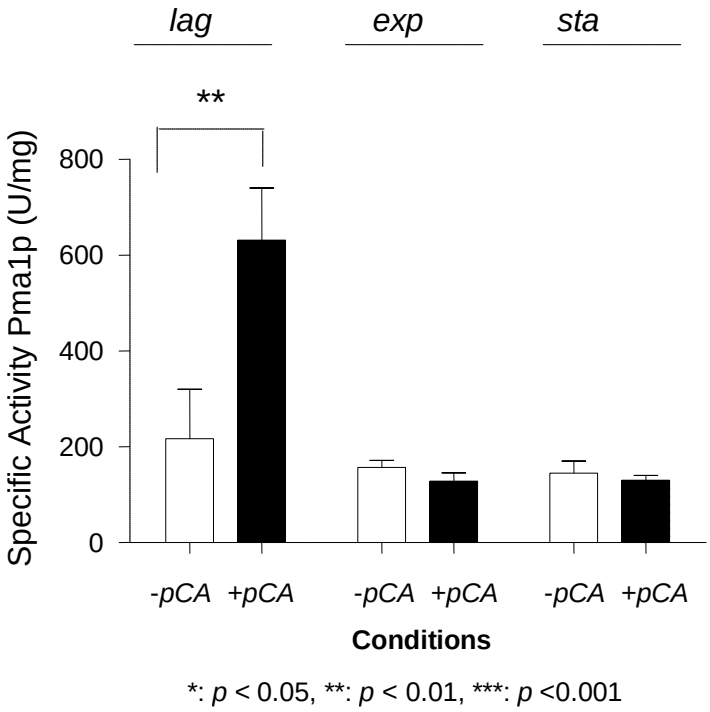

Figure 3. Specific activity of Pma1p during *lag (lag),* exponential (exp) and stationary (sta) of *D. bruxellensis* L1359 grown in SD media (open column) and in SD media containing *p*-coumaric acid at 100 mg/L (black column). All the experiments were conducted in triplicate. Data was analysed statistically using the student-t test with a level of significance of p<0.05.

containing phosphate groups (see materials and methods). During *lag* phase *D. bruxellensis* showed increased Pma1p activity in media containing *p*-coumaric acid However, during exponential and stationary phases Pma1p activity was similar for both growth conditions (Figure 3).

On the other hand, the observed increase in activity may also be due to increased amount of protein, however more studies are needed to prove this hypothesis.

The H^+- ATPase pump Pma1p has been associated with cellular homeostasis regulating internal pH and therefore helping cell growth (Serrano, 1989). This H^+-ATPase plays a critical role in cell adaptation to stress conditions caused by weak acids such as sorbic acid (Holyoak et al., 1996), octanoic acid (Viegas et al., 1998), cinnamic acid (Chambel et al., 1999), succinic and acetic acids (Carmelo et al., 1997) and hydroxycinnamic acids (Harris et al., 2008, 2010).

It has been estimated that during *S. cerevisiae* growth the H^+- ATPase Pma1p pump uses 10-15% of the total ATP produced by the cell (Gancedo and Serrano, 1989). However, in the presence of weak acids, the activity of Pma1p increases in response to the reduction of internal pH, which is vital for re-establishing homeostasis. Since this process demands a large amount of energy, up to 60% of the total ATP produced (Serrano, 1989; Holyoak et al., 1996), the high energy demand reduces ATP concentration until growth is minimal (Holyoak et al., 1996; Piper et al., 1997). This could explain the longer *lag* phase observed for *D. bruxellensis* when grown in media containing *p*-coumaric acid. Therefore, our results suggest the existence of an early adaptation mechanism involving H^+- ATPase pump Pma1p, which deals with the inhibitory effects of *p*-coumaric acid and potentially other hydroxycinnamic acids.

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Full Length Research Paper

Virulence characteristics of *Escherichia coli* isolates obtained from commercial one-week-old layer chicks with diarrhea

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Ninety (90) strains of *Escherichia coli* were isolated from intestine with fecal contents and liver of seven days old commercial layers presenting diarrhea, and their pathogenicity was determined by *in vivo* inoculation into the air sacs of day-old chicks. The test revealed 44 strains with high and intermediate pathogenicity level that were analyzed by PCR for the presence of eight virulence genes, and their serogroups were indentified using a set of anti-O antisera. Results demonstrate that these isolated strains contained at least one of the eight genes searched and the majority of them (93.20%) possessed gene *iss*. Seventeen (17) different genetic patterns have been detected with 15 having combinations of two or more genes representing 70.45% of all analyzed strains. Eleven different serogroups were identified, and the highest frequent was O8 (15.89%). Results demonstrate that strains that harbor genes *iss* or *astA* and some that belonged to serogroups O133 and O142 may have been crucial for the pathogenesis in the studied chicks, since several of these strains were pathogenic. The obtained results demonstrated the importance of studies in *E. coli* of avian origin in regions engaged in intensive poultry industry, aiming at evaluating the predominant strains and also acquiring preventive measures to minimize losses due to colibacillosis.

Key words: avian pathogenic *Escherichia coli* (APEC), layer chicks, polymerase chain reaction (PCR), virulence genes

INTRODUCTION

Strains of avian pathogenic *Escherichia coli* (APEC) are responsible for both systemic and localised infections in poultry. The disease known as colibacillosis results in significant morbidity and mortality, causing financial losses to the poultry industry which produces meat and eggs worldwide (Barnes et al., 2003). The bacterium affects birds of all ages, but the susceptibility to and severity of APEC are greatest in young birds (Montgomery et al., 1999; Johnson et al., 2001).

At a commercial layer farm, day-old chicks can arrive already infected with APEC (Guastalli et al., 2010). The infection can take place while the eggs are still in the incubator with transovarian transmission from infected hens to eggs or through egg shells contaminated by faeces. The bacterium penetrates through the egg shell, reach its interior and infects the embryo. Infected chicks that survive the first four days may develop serious infections and have compromised development.

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Table 1. Virulence genes, oligonucleotide sequences (primers), genomic locations, amplicon sizes, encoded virulence factors and primer references used in this study[a].

Gene	Oligonucleotide sequence (5' to 3')	Genomic location	Amplicon size (bp)	Encoded virulence factor	Reference (primers)
*ast*A	F=TGC CAT CAA CAC AGT ATA TCC R=TCA GGT CGC GAG TGA CGG C	Chromosome	116	Heat-stable enteroaggregative toxin	Sanger et al. (1977)
*irp*2	F=AAG GAT TCG CTG TTA CCG GAC R=AAC TCC TGA TAC AGG TGG C	Chromosome	413	Protein of the iron-acquisition system by the bacterium	Janβen et al. (2001)
*pap*C	F=TGA TAT CAC GCA GTC AGT AGC R=CCG GCC ATA TTC ACA TAA	Chromosome	501	P fimbria	Franck et al. (1998)
vat	F=TCC TGG GAC ATA ATG GTC AG R=GTG TCA GAA CGG AAT TGT	Chromosome	981	Vacuolating toxin	Dozois et al. (1992)
*iuc*D	F=ACA AAA AGT TCT ATC GC TCC R=CCT GAT CCA GAT GAT GCT C	Plasmid	714	Aerobactin	Franck et al. (1998)
iss	F=ATC ACA TAG GAT TCT GCC G R=CAG CGG AGT ATA GAT GCC A	Plasmid	309	Protein for increased serum survival	Dozois et al. (1992)
tsh	F=ACT ATT CTC TGC AGG AAG TC R=CTT CCG ATG TTC TGA ACG T	Plasmid	824	Adhesin that is sensitive to temperature	Dozois et al. (1992)
cva A/B *cvi cva*C	F=TGG TAG AAT GTG CCA GAG CAA G R=GAG CTG TTT GTA GCG AAG CC	Plasmid	1181	Colicin V	Dozois et al. (1992)

[a]Adapted from Ewers et al. (2005) and Kawano et al. (2006).

Furthermore, they may remain carriers and spreaders of pathogenic strains of *E. coli* (Barnes et al., 2003).

Several APEC serogroups were related to avian colibacillosis in Brazil and worldwide. According to Gross (1994), the most common serogroups are O1, O2, O8, O15, O18, O35, O78, O88, O109 and O115. However, many other serogroups that are rare, previously unknown and unable to be typed were detected (Menão et al., 2002; Silveira et al., 2002).

Through molecular biology, genes proposed as virulence factor markers in APEC have been determined (Ewers et al., 2005). Table 1 describes these genes, their genomic locations and their encoded virulence factors.

Considering these facts, the present work aims to investigate the presence of *E. coli* in seven-day-old chicks with diarrhea and apathy that are raised on commercial layer farms, to characterise the pathogenicity levels of the isolated strains through inoculation into the air sacs of day-old chicks and to identify the serogroups and virulence genes of these potentially pathogenic strains.

MATERIALS AND METHODS

This work is in accordance with the Ethical Principles in Animal Experimentation, adopted by the Brazilian College of Experimentation (COBEA) and was also approved by the Ethics and Animal Welfare (CEBEA), from São Paulo State University Animal Experimentation Ethics Committee, protocol n0. 026702-08.

Origins of *E. coli* isolated

Isolation of *E. coli* for the study was performed in 20 flocks of 7-day-old commercial layer chicks (producers of table eggs). Samplings were performed from August of 2008 to March of 2009 at different farms in the region of Bastos in São Paulo State, Brazil. Fifteen chicks from each flock with clinical signs of disease (smaller chicks in the flock with diarrhoea and apathy) were selected. These chicks were taken to the Unit of Research and Development of Bastos, where they were euthanised by cervical dislocation (approved by the São Paulo State University Animal Experimentation Ethics Committee - CEBEA, protocol n. 026702-08). One pool of liver fragments (15 animals) and another of intestines (in separated vessels) were collected aseptically from each flock.

E. coli isolation and identification

To isolate *E. coli*, a pre-culture of organ fragments was developed in Brain Heart Infusion (BHI) at 37°C for 18 h. Thereafter, the cultures were plated onto Eosin Methylene Blue agar (EMB) at 37°C for 24 h. Between three and six colonies of each organ pool, lactose fermenters (metallic green) were isolated and inoculated into separate tubes containing triple sugar iron agar (TSI) and incubated at 37°C for 24 h. The colonies inoculated into TSI that showed typical *E. coli* behaviours, such as glucose and lactose fermentation with gas production and the absence of H_2S, were confirmed by a biochemical series based on citrate utilisation, indol

production, methyl red and Voges-Proskauer reactions (Koneman et al., 1997). The isolated *E. coli* strains were stored on Luria Bertani agar.

Pathogenicity test in day-old chicks

Ten day-old male chicks obtained from a commercial source were used to determine the pathogenicity of each isolate. For this purpose, *E. coli* isolates were cultivated in BHI broth (10 mL) for 18 h at 37°C. Chicks were challenged with 0.1 mL of the culture containing approximately 10^7 colony-forming units/mL (CFU/mL) by inoculation into the left thoracic air sac of each chick (Dho-Moulin and Lafont, 1982; Monroy et al., 2005).

Chicks inoculated with culture medium (BHI) alone and with *E. coli* K12 strain that belong to the culture collection of the Laboratory of Bacterial Antigens II of the Department of Microbiology and Immunology of the Institute of Biology of the University of Campinas (Campinas/SP-Brazil) at the same concentration, served as negative controls. A standard *E. coli* strain (EC55 - serogroup O1 that belongs to the culture collection of the Laboratory of Avian Disease of the University of São Paulo - USP (São Paulo/SP - Brazil) was used as a positive control of pathogenicity (Guastalli et al., 2010).

The chicks were observed daily for ten days, and the strains were classified according to the following mortality index: highly pathogenic (mortality $\geq$80%), intermediate pathogenicity (mortality >50% but <80%), low pathogenicity (mortality ≤50%) and non-pathogenic (no mortality) (Monroy et al., 2005).

Multiplex polymerase chain reaction (PCR)

The strains were analysed for the presence of the genes *ast*A, *iss*, *irp*2, *pap*C, *iuc*D, *tsh*, *vat* and *cvi-cva,* whose primers, amplicons and gene locations are shown in Table 1. The strains EC 29 (*cva-cvi+, vat+, tsh+, iuc*D+*, Irp*2+*, iss+*) and M64 (*ast*A+, *iss*+, *irp*2+, *pap*C+, *iuc*D+, *tsh*+ and *cvi/cva*+) were used as positive controls (Ikuno et al., 2006). These strains were isolated from layer hens and belong to the bacterial collection of the Laboratory of Immunology of the Instituto Biológico (São Paulo/SP, Brazil).

DNA template preparation was performed using the Wizard Genomic DNA Purification Kit (Cat.# A1120, Promega Corporation, Madison, WI) according to the manufacturer's instructions. Multiplex PCR reactions were performed according to Ewers et al. (2005) with slight modifications: a total of 2 µL of each DNA template were added to a mix containing 1.25 - 2.50 µL of each primer (0.5 - 1 µM), 1 µL of each dNTP (100 µM each); 2.5 µL of PCR buffer (10x), 4 µL of 25 mM $MgCl_2$ and 2.5 U of *Taq* DNA polymerase (Fermentas, Europe), with a final volume of 25 µL. Amplification was carried out using a GeneAmp 2400 PCR System Thermal Cycler (Perkin Elmer, Waltham, MA) with the following conditions: t1, 3 min at 94°C; t2, 30 s at 94°C; t3, 30 s at 58°C; t4, 3 min at 68°C (t2-t4, 30 repeated cycles) and t5, 10 min at 72°C. Analysis of the amplified products was performed by electrophoresis (50 V for 2.5 h) with a 1.5% agarose gel stained with ethidium bromide in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and using 100-bp DNA ladder (Fermentas, Europe). Gel images were recorded using an Alpha Imager Photodocumenter 1220 (Alpha Innotech Corp., San Leandro, CA) connected to a computer.

Serogroup determinations

Serogroup determinations were performed according to the microplate technique (Guinée et al., 1972; Blanco et al., 1992) using an anti-O antisera collection (O1 to O185) belonging to the Laboratory of Bacterial Antigens II of the Department of Microbiology and Immunology of the Institute of Biology of the University of Campinas (Campinas/SP-Brazil).

RESULTS

Out of 20 studied flocks, 19 tested positive for *E. coli*, and 90 strains were isolated. As illustrated in Table 2, the number of strains from each flock varied from one to six, pathogenicity test in 1-day-old chicks revealed that 23 (25.55%), 21 (23.33%), 23 (25.55%) and 23 (25.55%) isolated were of highly, intermediate, low pathogenicity and non-pathogenic, respectively. During this test, all chicks of the positive control group died, while all chicks of the negatives controls group remained alive until the end of the experiment.

Macroscopic lesions were observed on necropsy on the chicks that died after the fourth day of inoculation. The lesions with the highest frequencies were airsacculitis (involving both air sacs) and yolk sac infections. Occasionally, pericarditis, perihepatitis, peritonitis with fibrin deposits and enteritis were found. Clinical signs, including diarrhoea and compromised body development, were also observed in some chicks. Clinical signs and macroscopic lesions occurred more frequently among chicks inoculated with strains classified with high and intermediate pathogenicity levels.

The 44 strains with high and intermediate pathogenicity levels had their serogroups and virulence genes determined. Serogroup characterisation revealed 26 serogrouped strains, in which 11 different serogroups were identified: O8 (15.89%), O9 (2.27%), O15 (4.54%), O23 (9.08%), O64 (4.54%), O75 (4.54%), O83 (6.81%), O112 (2.27%), O133 (4.54%), O140 (2.27%) and O142 (2.27%). For 18 strains (40.90%), serogroups could not be determined.

All potentially pathogenic strains in the pathogenicity test in day-old chicks contained at least one of the eight studied genes. Table 3 presents the results according to the number of strains and percent occurrence of each virulence gene.

No strain possessed all eight studied virulence genes, but 31 (70.45%) possessed more than one virulence gene. Seventeen genetic patterns were identified, of which two possessed only one gene and fifteen possessed an association of two or more genes. More strains were noticed at genetic patterns P13 and P15. Table 4 presents the genetic patterns of the *E. coli* strains with high and intermediate pathogenicity levels, associated with the serogroups and organs in which they were isolated.

DISCUSSION

The pathogenicity of an *E. coli* strain is based on the presence and expression of potential virulence factors (Won et al., 2009). According to Mellata et al. (2003), a

Table 2. Results of pathogenicity tests in day-old chicks of strains isolated from seven days old commercial layers presenting diarrhea.

Flock of chicks	Number of *E. coli* isolates	Organ from *E. coli* isolates	Mortality index		Pathogenicity classification
			Number of birds dead/challenged	Mortality (%)	
A	1	Liver	8/10	80	High
	2	Liver	8/10	80	High
	3	Intestine	9/10	90	High
B	4	Liver	5/10	50	Intermediate
	5	Liver	7/10	70	Intermediate
	6	Intestine	8/10	80	High
C	7	Liver	4/10	40	Low
	8	Liver	8/10	80	High
	9	Liver	4/10	40	Low
	10	Intestine	7/10	70	Intermediate
	11	Intestine	5/10	50	Intermediate
	12	Intestine	6/10	60	Intermediate
D	13	Liver	9/10	90	High
	14	Liver	7/10	70	Intermediate
	15	Liver	2/10	20	Low
	16	Intestine	10/10	100	High
	17	Intestine	0/10	0	Non-pathogenic
	18	Intestine	10/10	100	High
E	19	Liver	1/10	10	Low
	20	Intestine	3/10	30	Low
	21	Intestine	8/10	80	High
	22	Intestine	1/10	10	Low
F	23	Intestine	10/10	100	High
	24	Intestine	9/10	90	High
G	25	Intestine	0/10	0	Non-pathogenic
H	26	Liver	0/10	0	Non-pathogenic
	27	Liver	0/10	0	Non-pathogenic
	28	Liver	1/10	10	Low
	29	Intestine	0/10	0	Non-pathogenic
	30	Intestine	0/10	0	Non-pathogenic
	31	Intestine	10/10	100	High
I	32	Liver	3/10	30	Low
	33	Liver	6/10	60	Intermediate
	34	Liver	0/10	0	Non-pathogenic
	35	Intestine	0/10	0	Non-pathogenic
	36	Intestine	0/10	0	Non-pathogenic
	37	Intestine	2/10	20	Low
J	38	Liver	7/10	70	Intermediate
	39	Liver	1/10	10	Low
	40	Liver	6/10	60	Intermediate
	41	Intestine	0/10	0	Non-pathogenic
	42	Intestine	9/10	90	High
	43	Intestine	0	0	Non-pathogenic

Table 2. Contd.

K	44	Liver	8/10	80	High
	45	Liver	8/10	80	High
	46	Intestine	10/10	100	High
	47	Intestine	5/100	50	Intermediate
M	48	Liver	3/10	30	Low
	49	Liver	0/10	0	Non-pathogenic
	50	Intestine	2/10	20	Low
	51	Intestine	6/10	60	Intermediate
N	52	Liver	0/10	10	Low
	53	Liver	0/10	0	Non-pathogenic
	54	Liver	1/10	10	Low
	55	Intestine	0/10	0	Non-pathogenic
	56	Intestine	0/10	0	Non-pathogenic
O	57	Liver	0/10	0	Non-pathogenic
	58	Liver	0/10	0	Non-pathogenic
	59	Intestine	0/10	0	Non-pathogenic
	60	Intestine	8/10	80	High
	61	Intestine	0/10	0	Non-pathogenic
P	62	Liver	1/10	10	Low
	63	Liver	6/10	60	Intermediate
	64	Liver	7/10	70	Intermediate
	65	Intestine	4/10	40	Low
	66	Intestine	6/10	60	Intermediate
	67	Intestine	5/10	50	Intermediate
Q	68	Liver	0	0	Non-pathogenic
	69	Liver	0	0	Non-pathogenic
	70	Liver	0	0	Non-pathogenic
	71	Intestino	4/10	40	Low
	72	Intestino	10/10	100	High
	73	Intestino	0/10	0	Non-pathogenic
R	74	Liver	0/10	0	Non-pathogenic
	75	Liver	6/10	60	Intermediate
	76	Liver	10/10	100	High
	77	Intestine	6/10	60	Intermediate
	78	Intestine	6/10	60	Intermediate
	79	Intestine	5/10	50	Intermediate
S	80	Liver	4/10	40	Intermediate
	81	Liver	2/10	20	Low
	82	Liver	10/10	100	High
	83	Liver	1/10	10	Low
	84	Intestine	10/10	100	High
	85	Intestine	10/10	100	High

Table 2. Contd.

	86	Liver	2/10	20	Low
	87	Liver	7/10	70	Intermediate
T	88	Liver	2/10	20	Low
	89	Liver	5/10	50	Intermediate
	90	Intestine	8/10	80	High
	[a]*E. coli* 55		10/10	100	High
	[b]*E.coli* K12		0	0	Non-pathogenic

[a]*E. coli* EC 55 strain was used as a positive control; [b]*E. coli* K12 strain was used as a negative control.

Table 3. Number of strains and percent occurrence of virulence genes in the analysed *E.coli* strains.

Gene	*iss*	*ast*A	*iuc*D	*irp*2	*cvi/cva*	*vat*	*tsh*	*pap*C
No. of strains	41	18	19	12	8	4	6	3
%	93.2	40.1	43.2	27.3	18.2	9.1	13.6	6.8

bacterium's ability to resist inhibitory sera factors allows it to escape the actions of the complement system and phagocytosis. During the process of infection, the presence of a virulence factor (encoded by the *iss* gene) associated with this ability is more strongly correlated with a bacterium's level of pathogenicity.

The *iss* gene has been detected with high frequency in APEC. In this study, 41 (93.20%) strains were found to possess *iss*+. These data are in agreement with previous studies that have found levels of *iss*+ ranging from 80 to 100% (Ewers et al., 2004; Zhao et al., 2005; Someya et al., 2007; Kwon et al., 2008).

E. coli epidemiology is complex and involves humans, animals, the environment and the interactions among these components. According to Ikuno et al. (2006), the presence of virulence genes associated with commensal *E. coli* strains may be used as an indicator of potential risks because these bacteria may be reservoirs of virulence genes. In the same study, which was performed with egg-producing layers with clinical signs of colibacillosis, the facility environment, water consumed by layers and egg shelters, the *iss* gene was found in 50% of *E. coli* isolates, being 25% in the organs, 12.5% in the egg shelters and the remaining in the water consumed by layers and environment.

Furthermore, the participation of identified virulence factor genes in colibacillosis development has been supported by epidemiological studies that have demonstrated a significantly higher frequency of these genes in isolates from sick birds as compared to isolates of faecal samples from healthy birds (McPeake et al., 2005; Vandekerchove et al., 2005). However, the frequen-cies of some genes have varied significantly among APEC studies.

The *ast*A gene, which encodes a heat-stable enterotoxin (EAST) found in diarrhoeagenic bacteria, was detected in 88.5% of APEC analysed by Someya et al. (2007), in 20% of strains analysed by Ewers et al. (2004) and in 17.8% in strains studied by Won et al. (2009). In this work, 40.1% of strains were *ast*A+. Similarly, conflicting results have been found regarding the presence of the gene iucD, which encodes aerobactin, which was found in 43.2% of analysed strains on this study. Someya et al. (2007) detected this gene in 100% of analysed APEC, Ewers et al. (2004) detected it in 78%, and Won et al. (2009) detected it in 47.5% of their studied strains.

The gene that encodes a temperature-sensitive haemagglutinin (*tsh*) was detected in a low percentage of strains in this study (13.6%) as compared to results obtained by Zhao et al. (2005) and Won et al. (2009), which found this gene in 46.3% of analysed strains. Some studies have detected an even lower percentage of *tsh*+ strains, such as the study by Ikuno et al. (2006), which found that only 10% of analysed strains were *tsh*+.

The gene that encodes fimbria P (*pap*C) has been less frequently detected, with 6.8% of strains testing positive. This percentage was considered low when compared with data obtained by Won et al. (2009), who reported the presence of this gene in 15.0% of analysed strains, and Ewers et al. (2004), who reported the presence of this gene in 22.7% of analysed strains. However, one report

Table 4. Genetic patterns, number of strains with high and intermediate levels of pathogenicity, isolation sites and serogroups.

Pattern	Genotype	No. of strains	Organ	Serogroup
1	*iss*+, *ast*A+, *iuc*D+, *irp*2+, *cvi/cva*+, *pap*C+, *tsh*+	2	Liver/Intestine	O8/NT[a]
2	*iss*+, *ast*A+, *iuc*D+, *irp*2+, *cvi/cva*+, *tsh*+	1	Liver	NT
3	*iss*+, *ast*A+, *iuc*D+, *irp*2+, *cvicva*+, *vat*+	1	Liver	NT
4	*iss*+, *ast*A+, *iuc*D+, *irp*2+, *vat*+, *tsh*+	1	Intestine	NT
5	*iss*+, *iuc*D+, *cvi/cva*+, *tsh*+	1	Liver	O133
6	*iss*+, *ast*A+, *iuc*D+, *irp*2+, *cvi/cva*+, *pap*C+	1	Intestine	NT
7	*iss*+, *iuc*D+, *vat*+, *tsh*+	1	Liver	NT
8	*iss*+, *irp*2+, *cvi/cva*+, *vat*+	1	Liver	NT
9	*iss*+, *ast*A+, *cvi/cva*+, *iuc*D+	1	Intestine	NT
10	*iss*+, *ast*A+, *irp*2+	2	Intestine	O9/NT
11	*iss*+, *ast*A+, *iuc*D+	2	Liver/Intestine	O23/O112
12	*iss*+, *ast*A+	5	Liver	O8/NT
13	*iss*+, *iuc*D+	8	Liver/Intestine	O23/O64/O83/140/O142
14	*iss*+, *irp*2+	3	Liver/Intestine	O75/NT
15	*iss*+	11	Liver/Intestine	O8/O15/O83/O133/NT
16	*ast*A+, *pap*C+	1	Intestine	O64
17	*ast*A+	2	Intestine	O8/NT

[a]Not typable.

did not find this gene in any analysed strains of layers with clinical signs of colibacillosis (Ikuno et al., 2006).

Several studies have sought to determine the serogroups involved in APEC. Dho-Moulin and Fairbrother (1999) associated the identification of serogroups O1, O2 and O78 with highly pathogenic strains. However, the results from the present study demonstrate that no potentially pathogenic strain belongs to any of these serogroups. Serogroups O8, O9, O15, O23, O64, O75, O83, O112 and O140, representing 81.8% of the serogrouped strains in this study, have been cited in studies from several different countries (Blanco et al., 1998; Silveira et al., 2002; Jeffrey et al., 2004; Rosario et al., 2004; Vandekerchove et al., 2005; Zhao et al., 2005; Guastalli et al., 2010) as being involved in avian colibacillosis in chickens.

Blanco et al. (1998) stated that in the last several decades, different studies have demonstrated great antigenic diversity among APEC, with three to five serogroups being predominant among studied strains. In this study, serogroups that appeared at higher frequencies were O8 (15.89%), O23 (9.08%) and O83 (6.81%). The highest prevalence was NT strains. According to Silveira et al. (2002), the diversity of serogroups involved with colibacillosis may reflect regional differences associated with the prevalence of different clonal groups of strains.

In Mexico, Rosario et al. (2004), in a work developed (in a commercial chicken incubator) with strains isolated from infertile eggs and yolk sacs of dead embryos, found the following serogroups: O8, O9, O15, O23, O83 and O112, representing 54.0% of the total characterised serogroups in our study. In Brazil, a study with day-old commercial layer chicks identified 14 different serogroups from *E. coli* strains isolated from livers (Guastalli et al., 2010). Four of these serogroups, O8, O15, O64 and O75, were also identified in this study. The involvement of these groups in the aforementioned studies suggests their participation in colibacillosis in young birds or at the embryonic development stage.

The great number of APEC strains that could not be serogrouped makes diagnoses based on serotyping difficult (Dho-moulin and Fairbrother, 1999). In this study, 18 strains (40.9%) were not typable, in accordance with descriptions in the literature that described non-typable percentages of 14.8-60% (Silveira et al., 2002; Vandekerchove et al., 2004; Monroy et al., 2005; Zhao et al., 2005; Johnson et al., 2008).

Strains that belong to serogroups O133 and O142, found in the present study, had high and intermediate levels of pathogenicity, respectively, causing 100 and 70% mortality, respectively, in inoculated chicks; however, these serogroups have not been previously reported as being involved in APEC pathogenicity.

These results demonstrate the diversity of serogroups and virulence genes involved in colibacillosis pathogennesis. Strains that present a single virulence gene and serogroups that have not been commonly identified may be fundamental to disease pathogenesis in the studied chicks. Strains with high and intermediate levels of pathogenicity have been found, demonstrating the importance of studies of *E. coli* of avian origin, particularly in regions that practice intensive poultry industry, to evaluate predominant strains and acquire preventive measures to minimise losses due to colibacillosis.

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Full Length Research Paper

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

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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*

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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.,

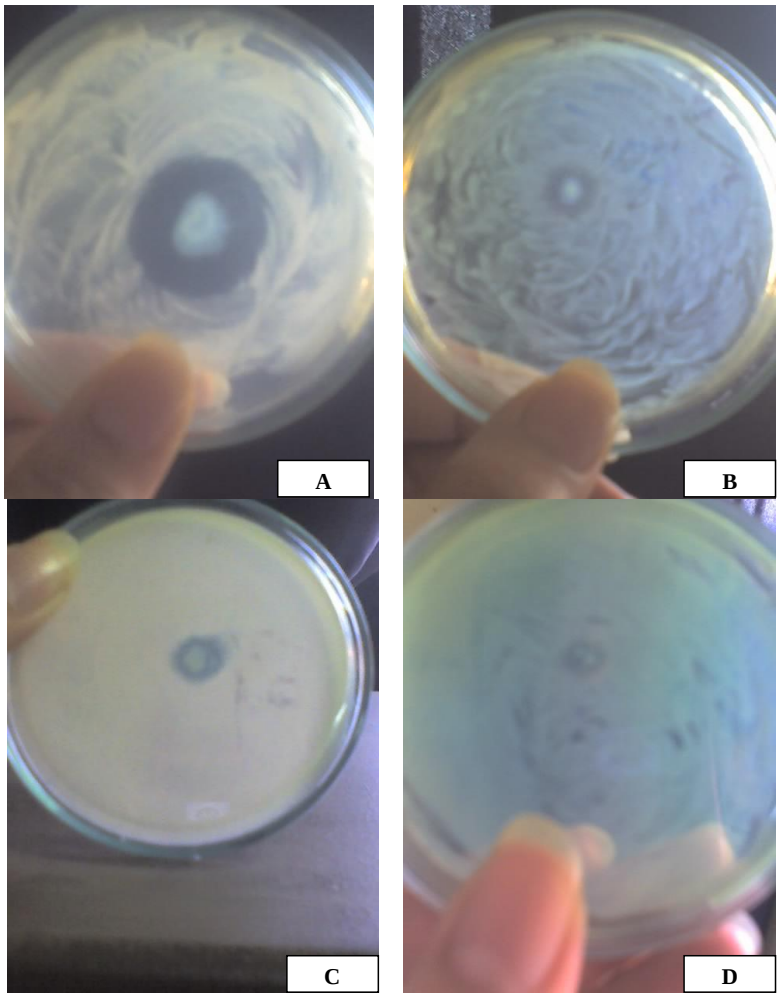

Figure 1. Inhibition zone of *A. faecalis* against *M. smegmatis* (A), *E. coli* (B), *B. subtilis* (C) and *P.aeruginosa* (D).Spot-on-lawn assay was performed to assess the antagonistic activity of *A. faecalis* against indicator bacteria. After incubation, the antibacterial effect was detected by the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

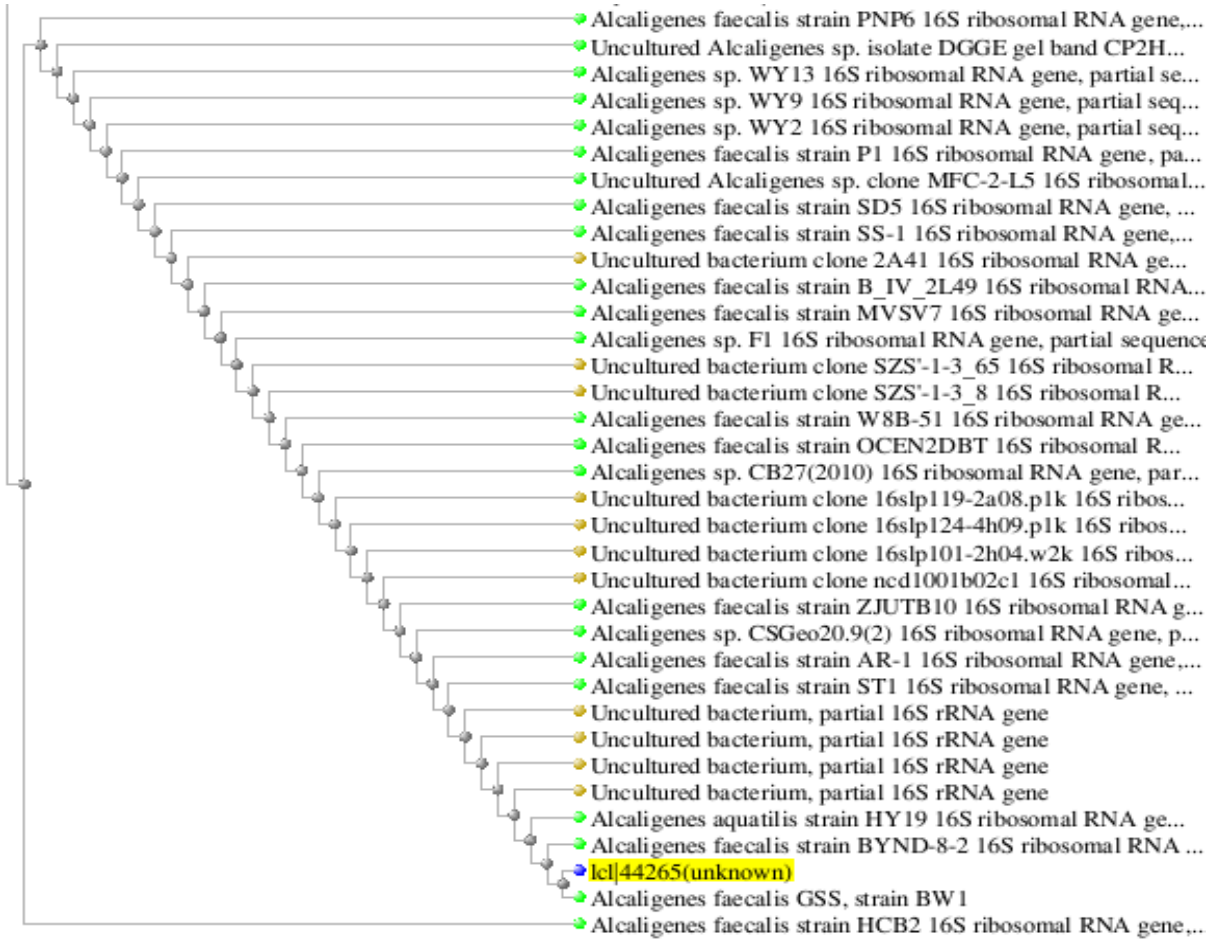

Figure 2. Phylogenetic tree showing the position of the isolate BW1. This microorganism is more closely related to *A. faecalis*. The position of BW1 is indicated by unknown in this figure.

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.

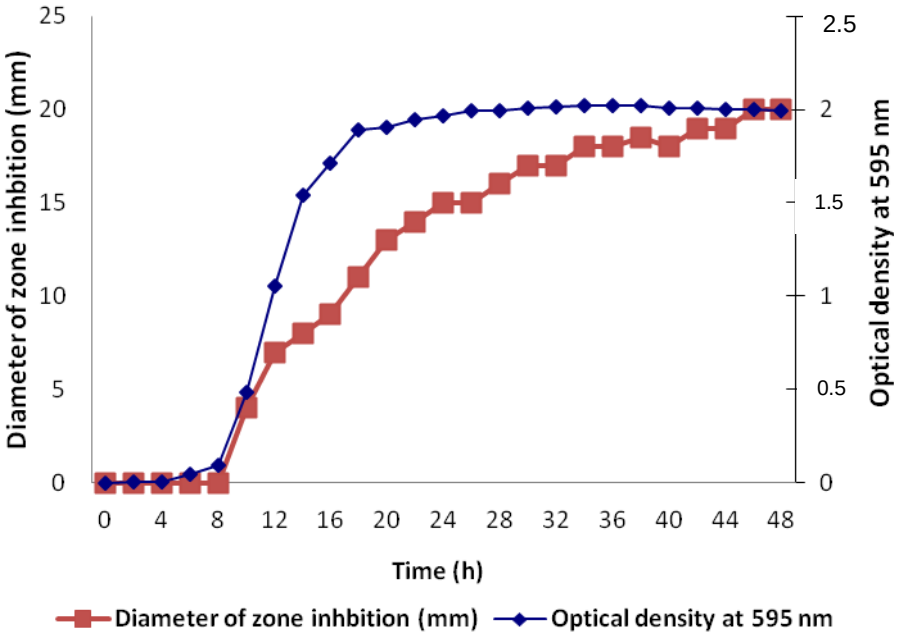

Figure 3. Growth kinetic and antibacterial agent production by *A. faecalis* (♦) growth kinetic, (■) antibacterial production. *A. faecalis* cells were cultured in LB broth and were incubated for 48 h at 37°C. The OD and antibacterial activity were measured.

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-

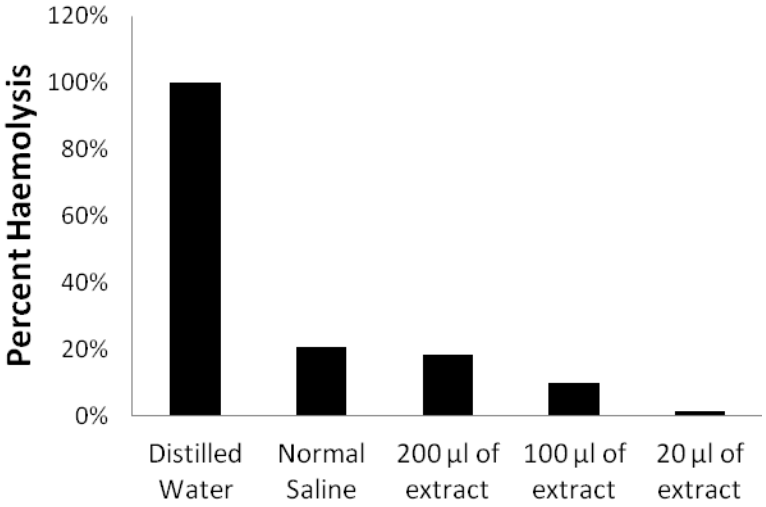

Figure 4. Cellular toxicity against human erythrocytes. Washed and diluted human erythrocytes in 0.9% NaCl solution were mixed respectively with different volumes of *A. faecalis* extract (20, 100 and 200 µl). The preparations were supplemented with 0.9% NaCl and incubated for 4 h at 37°C. The positive 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.

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African Journal of Microbiology Research

Full Length Research Paper

Phenotypic and genotypic characterization of methicillin and vancomycin resistant staphylococci

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One hundred (100) staphylococci were isolated from several Egyptian hospitals and laboratories which include 85 clinical isolates and 15 from hospital surroundings. The isolates were identified by conventional and molecular techniques. Fifty (50) isolates were identified as *Staphylococcus aureus* (SA), 40 were *Staphylococcus epidermidis* (SE) while 10 were identified as *Staphylococcus* species (SS). Upon testing the resistance to methicillin and vancomycin, it was found that resistance is dominant in isolates from clinical samples than those from the surrounding surfaces. Moreover, the resistance to methicillin was higher than that to vancomycin. Multiplex polymerase chain reaction was carried out to characterize the staphylococci-specific region of 16S rRNA gene, *mec*A gene associated with methicillin resistance and the virulence marker-associated genes Panton-Valentine leukocidin (PVL) *lukSlF-PV* genes which are responsible for leukocyte destruction and tissue necrosis. All the methicillin resistant staphylococci (MRS) were found to be *mec*A$^+$ while only five MRS carried *lukSlF-PV* genes. On the other hand, 30% of the methicillin sensitive staphylococci (MSS) were found to harbor the *mec*A gene while lacking the PVL. The results highlight the important role of horizontal gene transfer of virulence genes between staphylococci. In addition, this study indicates that the use of multiplex PCR is not sufficient for antibiotic susceptibility prediction and thus the simultaneous use of conventional and multiplex PCR technique is required for the identification of staphylococci and determination of their antibiotic susceptibility.

Key words: Methicillin resistance, *Staphylococcus aureus*, *Staphylococcus epidermidis, mec*A, Panton-Valentine leukocidin (PVL).

INTRODUCTION

Staphylococci are opportunistic human pathogen capable of causing a wide variety of diseases. The severity of a staphylococcal infection and its response to antibiotic treatment is dictated by the specific suite of virulence and antibiotic resistance associated genes (Peacock et al., 2002). Staphylococci impose challenge to clinicians, not only because of vancomycin and methicillin resistance, but also because of resistance to many other antibiotics (Levy and Marshall, 2004). Detection of methicillin resistance can be difficult due to the presence of two subpopulations, one susceptible and the other resistant that may coexist within a culture of staphylococci. This phenomenon is termed heteroresistance. Results using conventional phenotypic assay may be given after 48 h or more. In case of severe disease, the early detection of MRSA is essential. Thus detection of *mec*A gene using PCR is considered to be the best method (Prere et al., 2006).

Hospital associated methicillin resistant *Staphylococcus aureus* (HA-MRSA) acquired an integrated sequence into their genome (21-67-kb mobile genetic element), termed staphylococcal cassette chromo-some mec (SCC*mec*), which harbours the methicillin resistance gene *mec*A (Ito et al., 2001, 2004). The SCC*mec* chromosome contains

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*mec*A gene and two regulatory genes *mec*I and *mec*R1 which constitute *mec* complex that play an important role in the regulation of the *mec*A gene (Archer and Bosilevac, 2001; Berger-Bachi and Rohrer, 2002). Community-associated MRSA strains carry the Panton-Valentine leukocidin (PVL) virulence genes possessing a small mobile staphylo-coccal cassette chromosome *mec* (SCC*mec*) type IV or V genetic element which harbors the methicillin resistance (*mecA*) gene and which is more easily transferred to other strains of *S. aureus* than the larger SCC*mec* types (types I to III) that are prevalent in hospital associated MRSA strains (Vandenesch et al., 2003; Zhang et al., 2004).

McClure et al. (2006) developed a multiplex PCR to detect the staphylococci specific region of 16SrRNA gene along with virulence genes such as Panton-Valentine leukocidin (PVL) *lukS/F-PV* genes; which is responsible for leukocyte destruction and tissue necrosis and *mec*A gene associated with methicillin resistance. Concordance between phenotypic and genotypic characteristics was recorded.

The aim of this work was to determine whether the use of multiplex PCR amplifying these three genes would suffice the identification and prediction of virulence and the antibiotic susceptibility pattern of isolated staphylo-cocci.

MATERIALS AND METHODS

Bacterial isolates

A total of 100 clinical and hospital surroundings samples were collected from five different hospitals in Cairo. Samples from blood, urine, sputum, pus, throat, wound swabs, soil and patient beds were collected.

Samples were first collected on nutrient agar plates, purified and then sub-cultured on plates of blood agar, mannitol salt and Baird-Parker agar medium (Oxoid) using the streak plate method. The plates were incubated at 37°C for 24 - 48 h. Preliminary identification was carried out as recommended by Mahon and Manusekis (1995), Chapin and Lauderdale (2003) and Todar (2005). Gram stain, catalase and coagulase production were carried out to identify staphylococcal isolates (Cheesbrough, 1984; Koneman, 1992).

Antibiotic susceptibility test

Muller Hinton plates were inoculated with 0.5 McFarland standard inocula then different antibiotic disks were placed on the surface of the agar plates (methicillin 6 µg, ampicillin 10 µg, vancomycin 10 µg, linezolid 30 µg, clindamycin 2 µg, ciprofloxacin 10 µg, tobramycin 10 µg, erythromycin 15 µg, doxacycline 30 µg and cefoperaone 75 µg). The antibiotic susceptibility test was carried out according to Kirby-Bauer disk diffusion susceptibility test protocol (Bauer et al*.,* 1966) and the inhibition zones were measured as recommended by NCCLS (1997).

Determination of minimal inhibitory concentration (MIC)

Minimal inhibitory concentration of methicillin and vancomycin was tested according to Washington and Wood (1995) and NCCLS (1997).

Staphylococci are considered methicilin resistant (MRSA) if their MIC for oxacillin is ≥4 µg/ml and they are considered vancomycin resistant (VRSA) if their MIC for vancomycin ≥ 16 µg/ml (NCCLS, 1997).

Polymerase chain reaction

Genomic DNA was extracted from the bacterial isolates according to Sambrook et al. (2001). PCR were performed with three primer pairs. The first pair is *mec*A1 (5'-GTAGAAATGACTGAACGTCCGATAA-3') and *mec*A2 (5'-CCAATTCCACATTGTTTCGGTCTAA-3') corresponding to the *mec*A gene which encodes the unique penicillin-binding protein associated with oxacillin resistance in staphylococci (Zhang et al., 2004). The second pair is Staph756F (5'-AACTCTGTTATTAGGGAAGAACA-3') and Staph750R (5'-CCACCTTCCTCCGGTTTGTCACC-3') corresponding to regions of 16S rRNA genes that are unique to staphylococci (Zhang et al., 2004). The third pair is *LukPV*-1 (5'-ATCATTAGGTAAAATGTCTGGACATGATCCA-3') and *LukPV*-2 (5'-CATCAAGTGTATTGGATAGCAAAAGC-3') corresponding to the virulence marker (PVL) *lukS-PV* and *lukF-PV* genes that encode for tissue necrosis in staphylococcal infection (Lina et al., 1999). The PCR amplification products of the three primer pairs *mec*A, 16S rRNA and PVL genes are 310, 756 and 433 bp, respectively.

A routine laboratory PCR method for the detection of *lukS-PV* genes and *mec*A gene have required the use of separate PCR programs to investigate the proper conditions for each gene (Van Hal et al., 2007). Multiplex PCR conditions were performed according to McClure et al. (2006) and Van Pelt-Verkuil et al. (2008). One micro liter of DNA was added to 30 µl final reaction volume containing 3 µl primers mix (10 pmol specific for 16 srRNA, *lukS-PV* and *mec*A genes), 3 µl Taq Buffer (10x), 1.8 µl Taq Enzyme (25 Mmol mgcl), 3 µl (2Mmol DNTPase) and 13.7 µl nuclease-free distilled H_2O. The PCR was performed using AB Applied Biosystems thermocycler 2720. The PCR protocol was one denaturation step at 95°C for 5 min, followed by 25 cycle of denaturation at 95°C for 1 min annealing at 57°C for 1 min and primer extension at 72°C for 1 min, post extension step at 72°C for 10 min. Amplified PCR products were recognized in 0.8% agarose gel stained with ethidium bromide.

RESULTS

Eighty five (85) staphylococcal isolates were from clinical samples and 15 from hospital surfaces. Data were collected based on colony morphology, growth on blood agar media, mannitol salt agar media and Baird-Parker agar media, catalase and coagulase tests. Fifty isolates produced golden yellow colonies on mannitol salt agar medium and they were positive for catalase and coagulase. Thus, they were identified as *S. aureus* (SA). Forty isolates formed pink colonies on mannitol salt agar medium and were tested positive for catalase production but negative for coagulase and were identified as *Staphylococcus epidermidis* (SE). Ten isolates were atypical to either SA or SE. Therefore they were design-nated as *Staphylococcus* species (SS). The percentage of occurrence is shown in Table 1. The highest occur-rence of staphylococci was found in wound infections

Table 1. Sources and numbers of staphylococcal isolates, *S. aureus* (SA), *S. epidermidis* (SE) and unidentified *Staphylococcus* sp. (SS).

Source	Total number (%)	No. of SS isolates	No. of SE isolates	No. of SA isolates
Floor	6	1	2	3
Lab bench	3	0	1	2
Patient bed	6	1	3	2
Blood	9	0	4	5
Urine	19	2	10	7
Sputum	7	0	2	5
Pus discharge	12	2	3	7
Wound swab	21	1	7	13
Throat swab	11	1	6	4
Ear swab	3	0	1	2
Cerebrospinal fluid (CSF)	1	1	0	0
Pleural fluid	1	1	0	0
Cerebral fluid	1	0	1	0
Total no	100	10	40	50

Table 2. Distribution of methicillin resistant staphylococci (MRS) and vancomycin resistant staphylococci (VRS) according to their minimal inhibitory concentration (MIC).

Resistance (%)	(SA) (n=50)		(SE) (n=40)		(SS) (n=10)	
	Sur (n=7)	Clin (n=43)	Sur (n=6)	Clin (n=34)	Sur (n=2)	Clin (n=8)
MRS (MIC ≥ 4 µg/ml)	28.5%	53.5%	0%	41%	100%	100%
VRS (MIC ≥ 16 µg/ml)	0%	25.5%	0%	32.5%	0%	25%

followed by urine samples. In addition, SA strains were more dominant in wound infections while (SE) strains were more dominant in urine samples.

Antibiotic susceptibility test showed multi-drug resistance to tested antibiotics with various extents. Forty seven percent of all tested staphylococci were resistant to methicillin, 31% were resistant to vancomycin, 52% to ampicillin, 40% to ciprofloxacin, tobramycin and erythromycin, 36% to doxacyclin, 33% to clindamycin and 7% were resistant to linezolid.

MIC of methicillin and vancomycin were measured for all the staphylococcal strains. Strains able to grow at concentration 1 to <4 µg/ml were considered sensitive to methicillin (MSS) while methicillin resistant staphylococci (MRS) were those able to grow at concentration ≥4 µg/ml. Concerning MIC to vancomycin, vancomycin resistant staphylococci (VRS) were those that were able to grow at concentration ≥16 µg/ml. Isolates from the hospital surfaces were all sensitive to vancomycin. Staphylococci resistant to methicillin and vancomycin were more dominant in clinical isolates than the isolates from the hospital surface (Table 2). Moreover, the percentage of MRS was higher than that of VRS in clinical isolates. All tested resistant staphylococci showed high MIC values to both methicillin and vancomycin (Figure 1). Ten percent of both SA and SE were resistant to methicillin at concentration of 32 µg/ml. Moreover, 3 out of 50 (6%) of SA and 3 out of 40 (7.5%) of SE were resistant to vancomycin at concentration of 64 µg/ml. Fifty percent of SA and 35% SE strains were resistant to methicillin at concentration of ≥ 4 µg/ml. while 22 and 27.5% of the SA and SE strains were resistant to ≥16 µg/ml of vancomycin. Resistance of SS strains were relatively high as 70% of the strains were resistant to 16 µg/ml methicillin and 100% of the strains were resistant to ≥ 4 µg/ml, while 10% were resistant to 32 µg/ml vancomycin (Figure 1).

Single-targeted PCR products that were amplified from 16S RNA, *mec*A and PVL genes were 100% congruent with those targeting the three genes collectively in multiplex PCR. Multiplex PCR was carried out for all staphylococci isolates. All isolates were positive for the 16S rRNA gene specific for staphylococci. All MRSA and MRSE were found to have *mec*A gene. However, thirty percent of the phenotypically MSSA and MSSE were found to harbor *mec*A gene. Furthermore PVL gene was detected in five percent of the isolates. PVL positive isolates were SA strains recovered from wound infection,

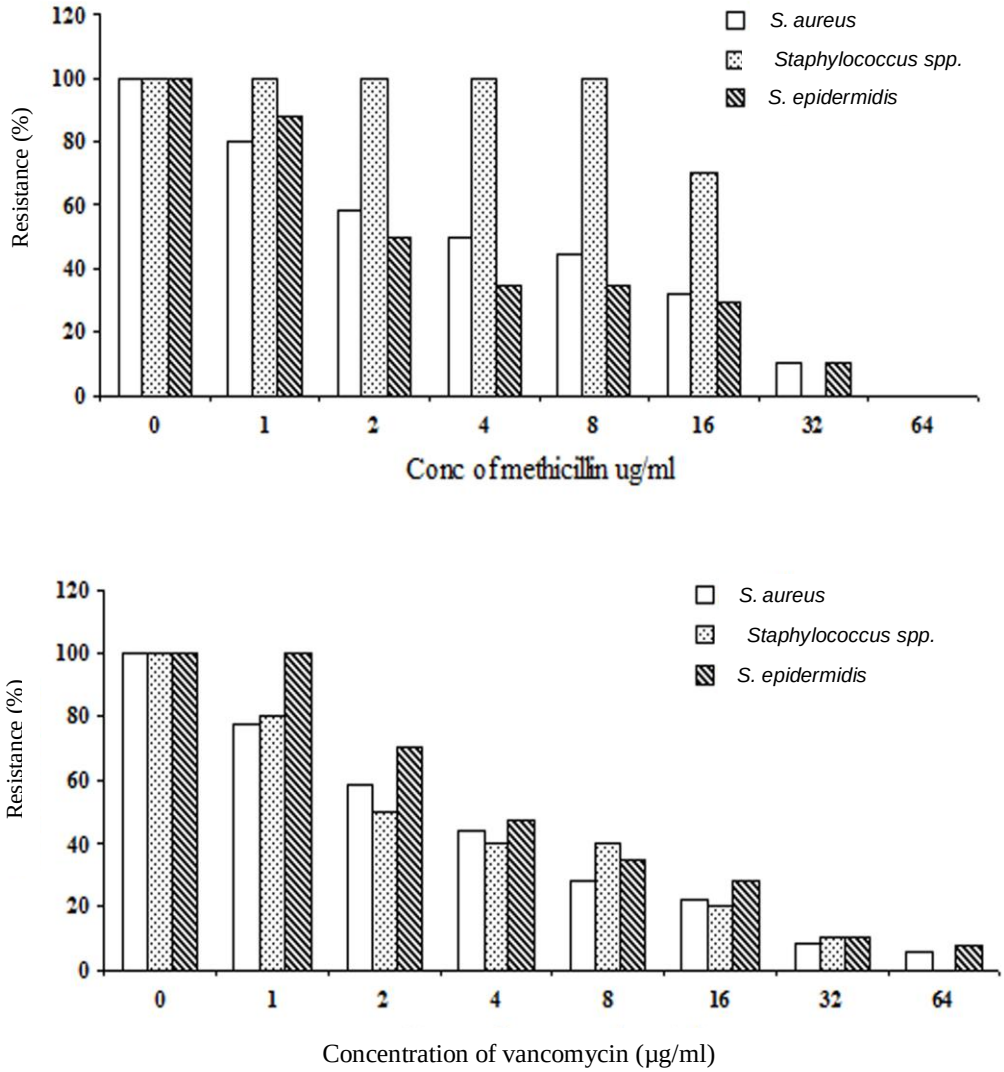

Figure 1. Percentage of methicillin and vancomycin resistance in staphylococci isolates recovered from their MIC data.

showing multidrug resistance and were *mec* A positive (Figure 2).

DISCUSSION

As a result of the extensive use of antibiotics, locally isolated staphylococci showed multidrug resistance to the tested antibiotics and also had relatively high MIC to methecillin and vancomycin. Braoios et al. (2009) and Mc clure et al. (2006) reported a correlation between the phenotypic and genotypic results for *S. aureus* as all MRSA had *mec*A gene while the MSSA lack the gene. In the present study, the MRSA and MRSE isolates were found to have *mec*A gene thus their phenotypic characterization were similar to their genotypic characterization both confirming their resistance. However, among the MSSA and MSSE, thirty percent of the isolates were found to contain *mec*A gene. The observation of phenotypically sensitive isolates which contain *mec*A gene highlights the possible role of horizontal gene transfer (HGT) in the dissemination of antibiotic resistance among MSSA strains. Many studies reported the transfer of *mec*A by HGT (Wielders et al., 2002; Hanssen et al., 2004). Several reports suggest that SCCmec transfer from methicillin resistant coagulase negative staphylococci (MR-CoNS) to methicillin-susceptible *S. aureus* (MSSA) occurs, although its mechanism remains unknown. MR-CoNS may thus act as a source of SCCmec for MRSA (Barbier et al, 2010). It could be concluded that phenotypic MSS might have partial SCC*mec* that lack the regulatory genes or it could have a mutated SCC *mec*. Further investigation is needed to determine the presence of unexpressed *mec*A gene in

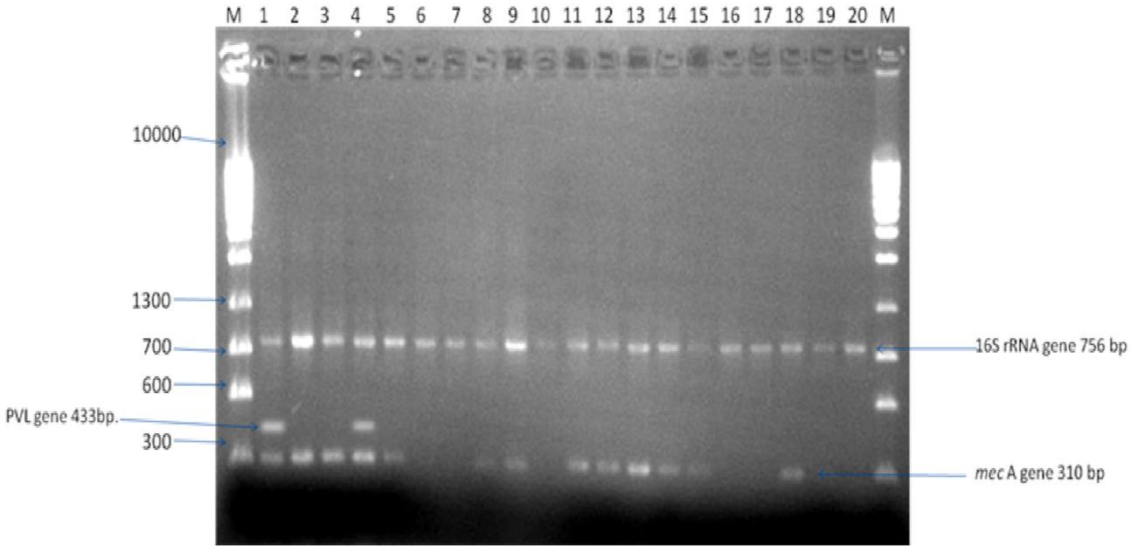

Figure 2. A representative agarose gel electrophoresis of multiplex PCR product resulting from amplification of genomic DNA using the three primer pairs of *mec*A gene (310 bp), specific region of 16S rRNA gene (756 bp) and the virulence marker PVL (*Luk s/f-pv*) genes product (433 bp), M is 1 kbp; DNA marker. Lanes 1-5 PCR products of resistant *S. aureus*, lanes 6-10: PCR products of sensitive *S. aureus*, Lanes 11-15 PCR products of resistant *S. epidermidis* and lanes: 16-20 PCR products of sensitive *S. epidermidis*.

MSS strains. Consequently, conventional methods for the detection of MRS by disc diffusion method are not sufficient for judging resistance (Fluit et al., 2001).

Multiplex PCR is an accurate method for the detection of the resistant and virulent isolates of staphylococci and it is recommended for a rapid detection and diagnosis (McClure et al., 2006). The choice of PVL genes is based on the finding that staphylococci which contain *(luk S-F pvl)* genes showed increased disease severity of cutaneous infection (Vandenesch et al., 2003; Deurenberg et al., 2004). Furthermore, it was reported that (*luk S-F pvl*) genes were found in a high proportion (77%) in emerging community acquired staphylococcal strains (Naimi et al., 2003; Shukla et al., 2004; Naas et al., 2005).

However the present study showed that only 5% of the examined isolates harbored the PVL. The PVL^+ isolate was recovered from pus discharging wound. The antibiotic resistance pattern of these isolates was similar to the other isolates lacking (*luk S-F pvl*) genes, thus indicating that the presence of (*luk S-F pvl*) genes does not necessarily indicate higher potency. Similar results were reported in a study where PVL^+ strains were compared with PVL^- mutant strains, both were found equally lethal and virulent (Said-Salim et al., 2005; Diep et al., 2006). The coexistence of PVL and *mec*A gene was demonstrated in only 5 out of the hundred examined isolates; this is in accordance with the results described by Gillet et al. (2002), Vandenesch et al. (2003) and Zhang et al. (2011) who concluded that PVL locus is carried on bacteriophage that is presently found in small proportion (less than 5%) of staphylococcal isolates described worldwide. Accordingly, the previously described principle that PVL is always associated with virulent staphylococci and result in out breaks in soft tissue is still debatable (Zhang et al. 2011). Further studies are required to draw more conclusions regarding the potential association between *mec*A, PVL and severity of infection. A combination of conventional and multiplex PCR is essential for the detection of the potentially pathogenic *Staphylococci* including phenotypically resistant and sensitive isolates.

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African Journal of Microbiology Research

Full Length Research Paper

Water condition and identification of potential pathogenic bacteria from red tilapia reared in cage-cultured system in two different water bodies in Malaysia

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The need to conduct periodic surveillance on the presence, associated pathological alteration in tissues and the various environmental factors that could trigger some potential fish pathogens that result to disease outbreak in tilapia farming in Malaysia is paramount. This study was conducted to determine the presence of potential pathogenic bacteria in fish and water bodies that could trigger disease outbreak. Some potential pathogenic bacteria were isolated and identified from water, sediments and tissues of red tilapia reared in cage-cultured system in Kenyir Lake, Terengganu and Semantan River, Pahang, east of Peninsular Malaysia, also the water quality was assessed using standard techniques. The brain, eye and kidney were collected randomly from 30 tilapias from each of these water bodies. The bacteria were isolated and identified using standard methods. In Kenyir Lake, bacterial isolates that predominated in selected tissues of tilapia were *Micrococcus* spp., *Aeromonas hydrophila*, *Staphylococcus* spp., *Pseudomonas aeruginosa* and *Enterobacter cloacae* while in Semantan River, *A. hydrophila* and *Staphylococcus* spp. predominated. The water quality of Semantan River was found to be above the recommended limits of ammonia, sulphide, iron and nitrite-nitrogen levels. For the water sample, *Staphylococcus xylosus* was the most predominant bacteria isolates in Kenyir Lake, while *Staphylococcus lentus* was the most predominant of Semantan River. From the sediments, *Pseudomonas aeruginosa* and *Enterobacter cloacae* were isolated in Kenyir lake while, *A. hydrophila* was found in Semantan river. From this investigation, *A. hydrophila* and *Staphylococcus* spp. are the predominant bacteria in Red hybrid tilapia; water quality, animal and human activities may play a role in the susceptibility of red tilapia to these potentially pathogenic bacteria which have not being previously observed in Malaysia. There is need for periodic surveillance of water, sediment and tissues of fish to detect the pathogens of paramount importance to Malaysian aquaculture industry.

Key words: Bacteria, Red hybrid tilapia, analytical profile index (API) test, water quality, lake, river.

INTRODUCTION

Red hybrid tilapia (*Oreochromis* sp.) was first introduced into Malaysia in the mid 1980's. It was initially considered to be hardy and resistant to diseases (Siti-Zahrah et al., 2004; 2008). However, unlike higher vertebrates, fishes being less immuno- competent could be predisposed to innumerable disease outbreaks (Bowser et al., 1998).

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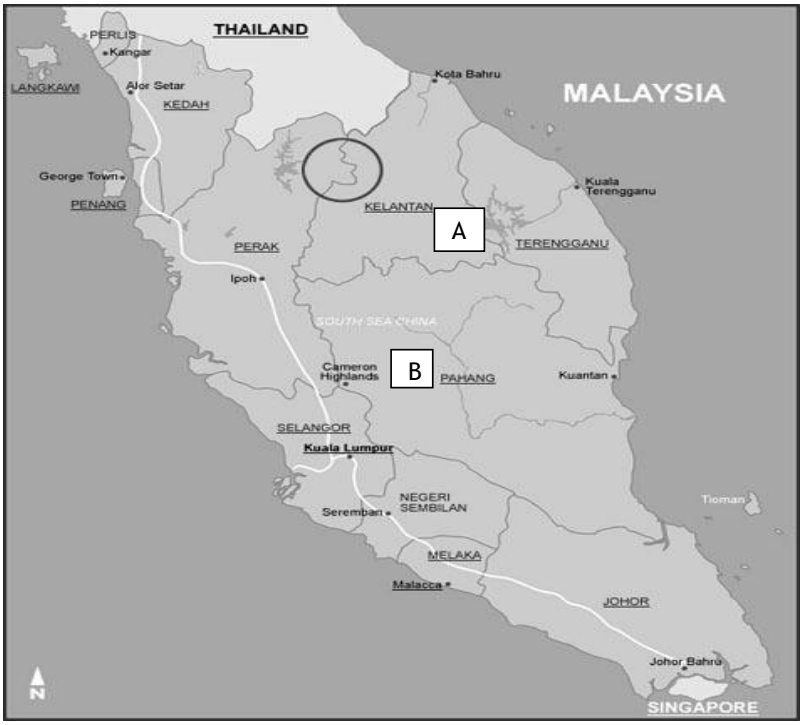

Figure 1. The study sites; A Kenyir Lake and B Semantan River.

Outbreak of bacterial diseases in fish remains one of the most significant limiting factors affecting fish culture worldwide (Zorrilla et al., 2003). The bacteria that had been associated with fatal outcomes in tilapia were *Aeromonas* spp., *Pseudomonas* spp., *Vibrio* spp., *Streptococcus* spp., *Enterococcus* spp., *Micrococcus* spp., *Staphylococcus* spp., *Plesiomonas* spp., *Moraxellaceae* and *Enterobacteriaceae* (Daskalov, 2006; Najiah et al., 2012). Of these, *Streptococcus* spp. had been the most important fish pathogen in Malaysia.

Since poor water condition including elevated nitrite and ammonia levels has been reported to be responsible for both morbidity and mortality in fish farming worldwide (Moraes and Martins, 2004), the need to conduct periodic surveillance on the presence, associated pathological alteration in tissues and the various environmental factors that could trigger some potential fish pathogens resulting to disease outbreak in Malaysian aquaculture industry is paramount. Thus, the study focuses on the isolation and identification of potential pathogenic bacteria from tissues of tilapia, water and sediments in two different water bodies. It also compared the water conditions from the cage-cultured of red tilapia in Kenyir Lake, Terengganu and Semantan River, Pahang in Malaysia.

MATERIALS AND METHODS

Location sampling and time

This study was conducted in cage-culture tilapia located in the east of Peninsular Malaysia. The most popular fish among the farmers were the Red hybrid tilapia. Sampling 1 was done at Kenyir Lake (5.00916°, 102.63310°), Hulu Terengganu, State of Terengganu and sampling 2 was located at Semantan River (3.48786°, 102.25317°), Temerloh, State of Pahang (Figure 1).

These locations were the commonly used for tilapia farming in Malaysia. Kenyir Lake is an artificial lake located in the state of Terengganu in northeast Malaysia, created in 1985 by the damming of the Kenyir river to create the Sultan Mahmud Power Station. The 260 km lake is reputed to be the largest artificial lake in Southeast Asia and it serves as a gateway to Taman Negara and Thailand.

Semantan River in State of Pahang is the longest river in in the Peninsular Malaysia with a length of about 435 km Pahang River. The climate is of equatorial type where the temperature is uniform throughout the year with temperature ranging from 21 to 32°C.

The humidity is consistently in the region of 75 to 80% throughout the year with an annual rainfall average of around 200 to 300 cm. The weather can be divided into dry (mid of year) and wet season (end to early year). Samples were collected during the wet season (rainfall season) for this investigation.

Fish, water and sediment

Thirty (30) fish were randomly selected per collection point. All the fish were measured and weighed. The external observations of the fish were also recorded. Also, ten samples of water and sediments were also collected in each site. Water samples were collected in 15 ml of sterile Bijou bottles under the water surface with the depth of 10 to 15 cm in different location within the cage-cultured farm from the lake and river.

All the samples were kept in the sterile containers and preserved in low temperature with icepack.

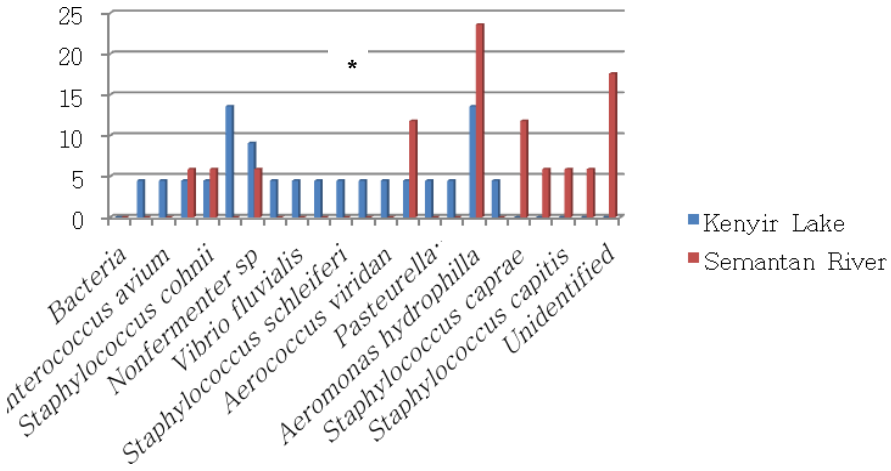

Figure 2. The predominant bacteria isolated from tilapia in two different water bodies. The asterisk shows that *A. hydrophila* was significantly higher ($P< 0.05$) in Semantan River as compared to Kenyir Lake.

Bacterial isolation and media

The fish were sacrificed and organs abnormalities were recorded. Brain, eyes and kidneys of each fish were collected for bacteriological analysis. Similar bacteriological analysis was done on the samples of water and sediments. Blood agar (BA) (Oxoid, UK) was used for the isolation before further characterization. All BA plates were labelled and sealed with adhesive tapes. The inoculated BA media were incubated at room temperature, ~22°C for 18 - 24 h.

These inoculated BA media were observed for the presence of bacterial growth after 24 h of incubation. After taking note of cultural growth characteristics, positive cultures were subjected to Gram's staining properties and cellular morphology observed with a light microscope (100x). Mixed colonies and Gram negative bacteria were subcultured on onto Trypticase Soya Agar (TSA) medium. Pure cultures of single colony type were transferred onto nutrient agar slants for a series of biochemical tests including catalase, oxidase and fermentative/ oxidative tests for identification following standard procedures (Austin and Austin, 1999). The bacterial colonies were then subcultured onto the BA media again to obtain the pure colonies of bacteria for analytical profile index test (API) (Morrison and Tillotson, 1988).

Biochemical test

Gram staining was done using the pure culture of bacterial colonies growth from TSA medium. For Gram-negative bacteria, API 20E was used including motility test, MacConkey medium bacterial inoculation, oxidase test, fermentation of glucose (OFF) and oxidation of glucose (OFO) to identify species of bacteria present. For Gram-positive bacteria, catalase test was done. Catalase positive by Gram-positive bacteria were subjected to API 20 Staph test. Catalase negative by Gram-positive bacteria will be further tested with API 20 Strep test. Catalase and oxidase tests were further used for the bacterial colonies from TSA medium. All the API test kit used was incubated in normal incubator at 30°C for 24 h. Identification of bacteria was done by using API test software.

Water quality measurement

The temperature, pH and dissolved oxygen and conductivity were measured using YSI 556 (YSI, USA). The ammonia, iron, sulphate and nitrate were determined using a DR 2800 Portable Spectrophotometer (Hach Company, Loveland, CO, USA).

Statistical analysis

Statistical analyses were performed using MedCalc for Windows, version 12.5.0.0 (MedCalc Software, Mariakerke, Belgium) and tested at 5% level of significance. The water conditions were analyzed using a one-way ANOVA and post-hoc test was administered using the Student-Newman-Keuls pairwise comparison test. The differences in other parameters were the figures.

RESULTS

Water quality

The water quality parameters (mean ± SD) were: dissolved oxygen, 4.55 ± 0.3 mg/L; temperature, 31.6 ± 0.8°C; pH, 7.40 ± 0.1; total ammonia, 2.40 mg L^{-1} and nitrate, 0.021 mg/L. All the parameters were within the normal range (El-Sayed, 2006)

Bacterial isolation

Figure 2 show the bacterial isolates from tilapia of Kenyir Lake which are mostly predominated by *Micrococcus* spp. and *Aeromonas hydrophila* with percentage of 13.64% of both bacteria and followed by non-fermenter sp. with 9.1%. In samples from Semantan River, the most predominant bacterial isolates are *A. hydrophila* with 23.53%, followed by *Staphylococcus xylosus* and *Staphylococcus caprae* both with 11.8%.

Water samples

In Kenyir Lake, the most predominant bacteria isolates

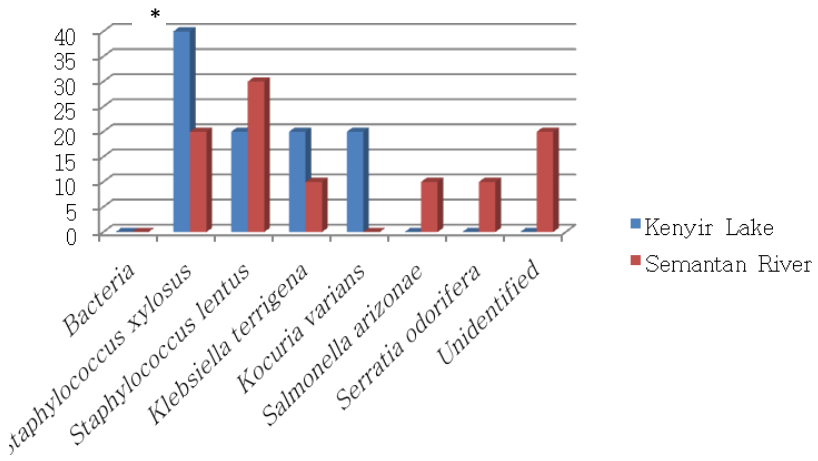

Figure 3. The predominant bacteria isolated from the water in two different water bodies. Asterisk shows the bacteria that were significantly higher (P< 0.05) in Kenyir Lake as compared to Semantan River.

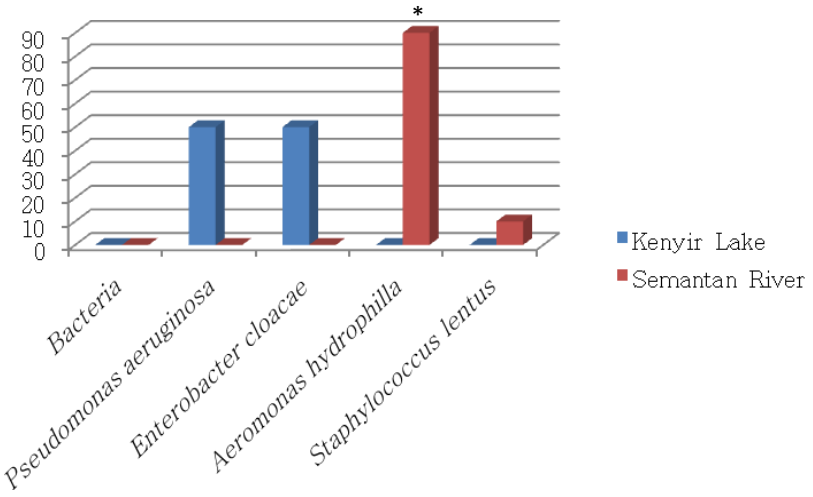

Figure 4. The predominant bacteria isolated from the sediment in two different water bodies. The asterisk shows the potential pathogenic bacteria which was significantly higher (P< 0.05) in Semantan River as compared to Kenyir Lake.

were *S. xylosus* (40%), followed by *S. lentus*, *Klebsiella terrigena* and *Kocuria varians* (20%), respectively. In Semantan river, *S. lentus* is the most predominant bacterial isolates (30%), and then followed by *S. xylosus* (20%). Meanwhile, the other bacterial isolates found are *K. terrigena, Salmonella arizonae* and *Serratia odorifera* which were 10%, respectively (Figure 3).

Sediment samples

There were two species of bacterial isolates isolated in Kenyir Lake, which are *Pseudomonas aeruginosa* and *Enterobacter cloacae* with 50%, respectively. About 90% of bacterial isolates in Semantan River are *A. hydrophila* and the remaining percentage are *S. lentus* (Figure 4).

Weight and length

The mean body weight of fish from Kenyir Lake, was significantly lower (P< 0.05) than that of Semantan River (Figure 5). However, both water bodies had about the same length of the fish (Figure 6).

Water quality

There was no significant difference (P> 0.05) in the water

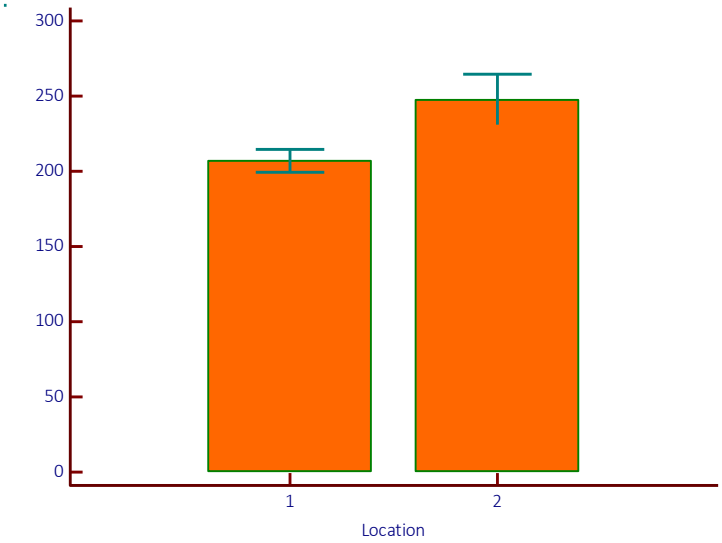

Figure 5. The body weight of tilapia from two different types of water bodies. Semantan River shows tilapia had similar body weight with that of Kenyir Lake.

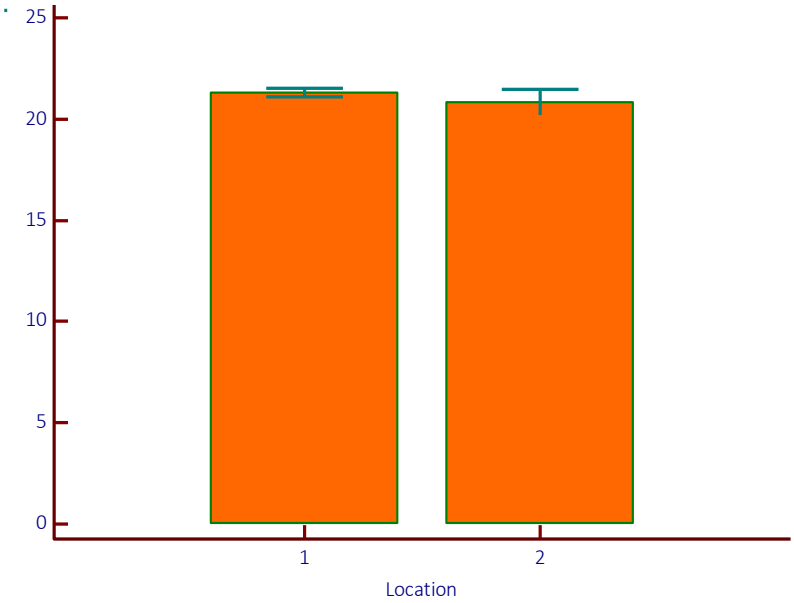

Figure 6. Tilapia from different types of water bodies showing similar length.

quality (pH and conductivity) between Kenyir Lake and Semantan River. Of the parameters measured, sulphide, Fe and NH_3 in Semantan River were significantly higher ($P< 0.05$) than that of Kenyir lake while temperature, DO and NO_2:N (Figure 7) were similar.

DISCUSSION

This investigation describes the isolation and identification of potential fish pathogens in water, sediments and tissues of Red hybrid tilapia reared in cage-cultured system in two different water bodies in Malaysia.

The predominant bacteria isolated from tissues of Red hybrid tilapia from Kenyir Lake includes *Micrococcus* spp. and *Aeromonas hydrophila* while that of Semantan River, are *Aeromonas hydrophila, Staphylococcus xylosus* and *Staphylococcus caprae*. This showed that the bacteria isolated from fish vary with the water source and pollution level. The predominant bacteria from the two study sites are *A. hydrophila*, which has been reported by other workers as part of the normal flora of freshwater fish

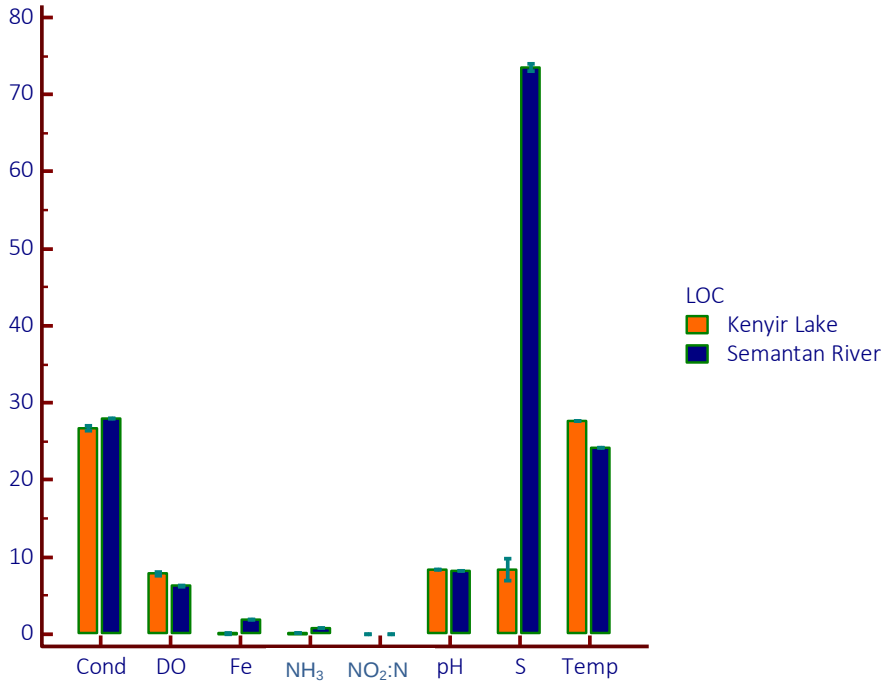

Figure 7. Water conditions in Semantan River and Kenyir Lake.

(Sugita et al., 1982; Santos et al., 1998; Thayumanavan et al., 2003). In this study, the tilapia that *A. hydrophila* were isolated showed no clinical signs, however at necropsy, there were evidence of renal hypertrophy and congestion, encephalomalacia and hepatomegaly. These findings are in agreement with the reports of other workers (Ghosh and Homechaudhuri, 2012). Since *Aeromonas* spp. had been reported to be an opportunistic pathogens that do invade heavily stressed fish (Inglis et al., 1993; Saha and Pal, 2000), the high mortality observed in Semantan River during sampling and isolation of *A. hydrophila* from the fish suggested *A.hydrophila* as a possible pathogenic bacterium that could lead to clinical diseases of stressed Red tilapia in Malaysia as earlier reported elsewhere (Lio-Po et al., 1983). It should be noted that in this present study, *Streptococcus agalactiae* was not isolated from the samples despite the reports of heavy mortality associated with outbreak of the disease in Malaysia (Amal et al., 2008). This could be associated with the reports of Siti-Zahrah et al. (2004, 2008) that *Micrococcus* and *Staphylococcus* spp. are the predominant bacteria often isolated in Kenyir Lake.

Micrococcus spp. infected fish showed no clinical sign but gross lesions include swollen or congested kidney and pale liver. The occurrence of lesions without any clinical signs further strengthens the need for periodic surveillance of this nature that will help in the detection of subclinical infections.

The presence of *Staphylococcus caprae* from Semantan River fish further showed that the farmer uses goat faeces as fish feed. This act should be with caution as there had been reports of bacteria isolated primarily from goats being pathogenic to fish (Carretto et al. 2005)

S. xylosus found in this study had also been previously reported (Siti-Zahrah et al., 2008). However, *S. xylosus* and other bacteria such as *Staphylococcus chromogens*, *Staphylococcus warneri*, *Staphylococcus capitis* and *Staphylococcus cohnii* had been reported to infect sea bream and sea bass in Greece causing dark body, fin and skin necrosis to shallow ulcers, hence isolating this bacteria from fish are of great concern, more importantly when infected fish showed no clinical sign except for a few with pale liver and congested kidney. The isolation of *Pasteurella multocida* from fish with no clinical sign and lesion showed that the infection is subclinical but worthy of note is the outbreak of pasteurellosis in tilapia hybrids from a fish farm on the shore of lake Kinneret, Israel (Nizan and Hammerschlag, 1993) and the source of infection was suggested to be the chicken manure used as organic fertilizer in the ponds. The possible transmission and pathogenicity of this bacterium in fish need to be investigated.

The presence of *Enterococcus avium* in fish may originate from contaminated water through the excretion of animals and humans waste. *Enterococcus* sp. had been reported to cause considerable economic losses in cultured turbot (*Scophthalmus maximus*) (Toranzo et al., 1995) and tilapia (Plumb, 1999). However, the infected fish also showed no apparent clinical signs or lesions.

Infected fish with *Vibrio fluvialis* also did not show clinical signs but only a pale liver and hyper-pigmentation of the eye. The detection of this bacterium in fish from the study sites is also of great concern as *V. fluvialis* have been reported to be pathogenic for tilapia hybrids (Xu et al., 1993) in Japan and Israel with haemorrhages around the base of the fins, prostration in the swimming movement and stiffness of the muscles (Bisharat et al., 1999).

S. epidermidis has been previously isolated from tilapia in Malaysia (Siti-Zahrah et al., 2008) but infected fish also did not show any clinical signs except a pale liver and a congested kidney. *Aerococcus* sp. and *S. aureus* had also been isolated from freshwater fish by other workers (Siti-Zahrah et al., 2008). The infected fish showed a congestion of the kidney and pale liver without the external lesion (Najiah et al., 2012). However, various reports abound on their pathogenicity in silver carp in India causing corneal damage (Shah and Tyagi, 1986). *Plesiomonas shigelloides* is a Gram-negative bacterium, though not specific for aquatic species but is widely distributed in water and soil in temperate and tropical regions hence it is considered as a potential fish pathogen (Cruz et al., 1986). The presence of *Salmonella arizonae* and *Enterobacter cloacae* in the fish tissue and in the water suggests pollution (Burras, 1993) possibly from contamination with animal waste from chicken as observed by Abd El-Aziz and Ehab (2003). *Pseudomonas aeruginosa* isolated from the debris of Kenyir lake, is a common bacterial pathogen of fish in most countries with remarkable septicaemia and haemorrhages in the skin of the mouth region, opercula and ventral side of the body (Buller, 2004).

Environmental stress has influenced the pattern of isolation and disease. The temperature of water in Kenyir lake was observed to be higher than Semantan River probably because lake tends to retain heat for a longer period, resulting to lower cooling, that could account for higher numbers of type of species of bacteria isolated since dissolved oxygen (DO) in the water column is one of the most important factor for maintenance of life of cultured organisms (Harada, 1978). Ammonia level in Kenyir Lake and Semantan river exceeded the acceptable limit (0.02 ppm) for fish. The high level of ammonia may be due to the high level of ammonia in the feed, where the goat faeces and visceral organs of chickens were used as a source of feed. The mortality of fish observed in Semantan River at the time of this study may be associated with the high level of ammonia. In Semantan River, the weight of fish was more than Kenyir Lake fish, and because the small-sized Nile tilapia had been found to be more tolerant to nitrite than larger fish (Atwood et al., 2001), it could explain the unusual high mortality observed with fish from Semantan River (Branson and Southgate, 1992).

In this investigation, more than one species of bacteria were isolated from the fish, which may be associated with the nature of feed, sediments and poor water quality observed. This passive surveillance of this nature is essential for the prevention of possible disease outbreak associated with the potential pathogenic bacteria observed especially during the critical period of environmental stress. The understanding of the virulence mechanisms of the pathogenic bacteria observed will aid the development of strategies for preventing or managing diseases caused by the potential fish pathogens in future.

ACKNOWLEDGEMENTS

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African Journal of Microbiology Research

Full Length Research Paper

Use of combination of bacteriocins from *Lactobacillus plantarum* MTCC 1407 and *Bacillus coagulans* MTCC 492

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Bacteriocins are antimicrobials produced mainly by lactic acid bacteria as well other genera, a property which can be exploited in food biopreservation. However, narrow spectrum of activity of these bacteriocins is a limitation for their use in different food systems. Various approaches are being pursued to increase their antimicrobial efficacy like use of increased dosage, use of highly purified form in combination with other preservative techniques such as HPP, ultrasonic waves, use of a combination of bacteriocins, etc. Bacteriocins from two producers namely *Lactobacillus plantarum* MTCC 1407 and *Bacillus coagulans* MTCC 492 were used in the present study. Partially purified form of bacteriocin produced by them was tested individually as well as in combination with antimicrobial activity in liquid medium against food spoilage agents, Gram positive organisms such as *Streptococcus thermophilus, Leuconostoc mesenteroides, Micrococcus flavus* and also Gram negative organisms such as *Escherichia coli and Pseudomonas aeruginosa.* They were all susceptible to antimicrobial action of these bacteriocins, 26 to 72% inhibition was recorded with turbidometric method of assessment of bacteriocin activity. However, bacteriocins when used in combination of 1:1 (v/v) did not result in increased inhibition.

Key words: Bacteriocins, synergy, *Lactobacillus* sp., *Bacillus* sp., food spoilage agents.

INTRODUCTION

Despite modern advances in technology, the preservation of food is still a debated issue in developing as well as industrialized countries. Alleviation of economic losses due to food spoilage, lowering the food processing costs and avoiding transmission of microbial pathogens through the food chain while satisfying the growing consumers demands for food that are ready-to-eat, fresh tasting, nutrient and vitamin rich and minimally processed and preserved are the major challenges of the current food industry (Mangaraj and Singh, 2008). Consumers are concerned with possible adverse health effects of the presence of chemical additives in their foods (Soomro et al., 2002).

Reduction or inhibition of unwanted food based microorganisms by biological means is thus gaining importance. Biopreservation refers to extended storage life and enhanced safety of foods using the natural microflora and their antibacterial products (Ross et al., 2002). Lactic acid bacteria have a major potential for use in biopreservation because they are safe to consume and during storage they naturally dominate the microflora of many foods. Lactic acid bacteria have been granted generally regarded as safe (GRAS) status (Stiles, 1996). Bacteriocins is their antimicrobial metabolite which has

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potential to control the growth of spoilage and pathogenic bacteria in foods (O`Sullivan et al., 2002).

The term 'bacteriocins' was coined in 1953 to define colicin produced by *Escherichia coli*. They are ribosomally synthesized, extra-cellularly released low molecular mass proteins which have bactericidal or bacteriostatic effect on other micro-organisms (Klaenhammer, 1988; Tagg et al., 1976) either of the same species or other genera (Cotter et al., 2005). The first bacteriocin was discovered in 1925 by Gratia (Garneau et al., 2002). Majority of bacteriocin producers are *Lactobacillus* spp., *Enterococcus* spp., *Pediococcus* spp. and *Leuconostoc* spp. (De Vuyst and Vandamme, 1994) but those produced by *Lactococcus*, *Streptococcus*, *Clostridium* and *Carnobacterium* were also described.

Applications of bacteriocins for the control of some pathogens and food spoilage organisms has been approved in a number of countries (Cleveland et al., 2001; O'Sullivan et al., 2002; Chen and Hoover, 2003; Cotter et al., 2005; Fimland et al., 2005; Deegan et al., 2006; Drider et al., 2006). Advances in bacteriocins research and combination treatment for food preservation will benefit both the producer and consumer. The only bacteriocin given GRAS status is nisin (Federal Register, 1988). It is commercially used in food systems (Settani and Corsetti, 2008).

Bacteriocins are suitable for food preservation and recent studies conducted suggest that their use offers a lot of advantages such as a) extend shelf life, b) provide protection especially during times of temperature abuse, c) decrease the risk of transmission of food borne pathogens, d) decrease the losses due to food spoilage, e) reduce the application of chemical preservatives, f) permit the application of less severe heat treatment without compromising food safety (Hurdle Concept). Bacteriocins are non-toxic to eukaryotic cells and hence pose no threat to human intestinal cells. Being proteinaceous in nature they are readily degraded by protelytic enzymes in human gastro-intestinal tract. Moreover, they do not have any therapeutic application and are not known to cause allergies. Being of LAB origin they are probiotic in nature and also help in restoring the normal gut microflora (Thomas et al., 2000).

Lactobacillus spp. is an important bacteriocin producer. Bacteriocins produced by them exhibit bactericidal mode of action (Klaenhammer, 1988). They are effective against *E. coli* (Lade et al., 2006; Torodov and Dicks, 2004; Caridi, 2002), *Acinetobacter baumanii* (Torodov and Dicks, 2005); *Aeromonas hydrophila* (Messi et al., 2001); *Listeria monocytogenes, Staphylococcus aureus, Enterococcus faecalis, E. coli, Yersinia enterocolitica* and *Yersinia pseudotuberculosis* (Miteva et al., 1998; Ennahar et al., 1996).

Bacillus spp. is comparatively a new entry to the list of bacteriocin producers. Their bacteriocins are less worked on as compared to bacteriocins from lactic acid bacteria. Most of them are lantibiotics (Abriouel et al., 2011). Production of bacteriocins has been detected in *Bacillus subtilis*, *Bacillus cereus*, *Bacillus stearothermophilus* and other Bacilli (Bizani and Brandelli, 2002). The bacteriocins produced by them have a broad spectrum, being effective against food borne pathogens such as *Streptococcus pyogenes* (Cherif et al., 2001). Lichenin produced by *Bacillus licheniformis* and megacin produced by *Bacillus megaterium* have been well characterized (Lisoba et al., 2006).

As stated earlier, bacteriocins have a narrow spectrum of activity. They are generally effective against closely related species (Cotter et al., 2005). They are effective against Gram negative organisms as well but only when used in high concentrations (Deegan et al., 2006). Also, most of them are ineffective or effective to a very less degree against yeast and molds which limits their use in food systems (Dalie et al., 2010). Efficacy of bacteriocins can be enhanced by using them in combination with other chemicals and other preservative techniques (Cleveland et al., 2001). Use of a combination of bacteriocins can broaden the antimicrobial spectrum and also prevent the emergence of bacteriocin resistant strains. The present investigation was carried out to elucidate the *in vitro* antimicrobial spectrum of bacteriocins produced by *Lactobacillus plantarum* MTCC 1407 and *Bacillus coagulans* MTCC 492 and investigate whether the use of a combination of them can be of advantage.

MATERIALS AND METHODS

L. plantarum MTCC 1407 and *B. coagulans* MTCC 492 were used as bacteriocin producers in the present study. The indicator organisms chosen were *Streptococcus thermophilus* MTCC 1928, *Leuconostoc mesenteroides* MTCC 107, *Micrococcus flavus* ATCC 10240, *E. coli* MTCC 1650 and *Pseudomonas aeruginosa* ATCC 10662. They were purchased from Microbial Type Collection Collection at Institute of Microbial Technology, India and maintained on their respective recommended media (Table 1).

The growth kinetics of the producer organisms was plotted spectrometrically at 600 nm and various growth phases identified. Bacteriocin production is related to growth phases of the producer. It was purified from broth culture at various stages of growth. Bacteriocin was purified by the method of Allende et al. (2007). Broth cultures were centrifuged at 5000 rpm for 10 min at 5°C using a cooling centrifuge (REMI C30). The supernatant was neutralized using 2N NaOH and then filter sterilized using membrane filters of pore size 0.22 µ. Subsequently it was heated at 80°C for 3 min. Lactic acid and other organic acids were neutralized by NaOH. H_2O_2 is degraded by heating to minimize the chances of getting a false positive result. This partially purified bacteriocin preparation was kept at -10°C throughout the time period of this study.

Bacteriocin assay was performed by turbidometric method of Turcotte et al. (2004) also known as percentage inhibition method with minor modifications. Suspension of metabolically active cells of the indicator organisms was prepared. It was calibrated to contain a population corresponding to McFarland Standard 0.5 (approximate cell density of 1.5×10^8 cells/ml). Bacteriocin preparation and indicator suspension were mixed in ratio 1:1 (v/v). Sterile broth was used as diluting medium to maintain the total volume of reaction mixture in the control run. The reaction mixtures were incubated for

Table 1. Details of microbial cultures used in the present investigation.

Organism	Recommended medium	Optimum temperature/pH	Incubation time (h)
Lactobacillus plantarum MTCC 1407	MRS	30°C/6.5±0.2	48
Bacillus coagulans MTCC 492	Nutrient Agar	37°C/7±0.2	24
Streptococcus thermophilus MTCC 1928	Brain Heart Infusion	37°C/7.0±0.2	48
Leuconostoc mesenteroides MTCC 107	MRS Agar	25°C/7.0±0.2	48
Micrococcus flavus ATCC 10240	Nutrient Agar	30°C/7.0±0.2	48
Escherichia coli MTCC 1650	Nutrient Agar	37°C/7.0±0.2	24
Pseudomonas aeruginosa MTCC 10662	Nutrient Agar	25°C/7.0±0.2	48

6 h (bacteriocin from *B. coagulans*) and 12 h (bacteriocin from *L. plantarum*). Taking A_m to be the absorbance of the sample recorded at 700 nm and A_o as absorbance of the control, percentage inhibition was calculated as per the formula:

Inhibition (%) = $1- A_m/A_o \times 100$

Use of combination of bacteriocins

Bacteriocin preparations from both organisms were mixed in equal amounts and assay performed as explained above against food spoilage organisms.

RESULTS AND DISCUSSION

The results presented are an average of multiple trials. The percentage inhibition mentioned is an average of at least five consistent recordings. As stated earlier, bacteriocin preparation prepared from one batch was used throughout the study. Initially, the various growth phases of the producer strains were indentified (data not shown). Bacteriocin was partially purified from various growth phases and assay was performed. Bacteriocin preparation exhibiting maximum inhibition was used for antimicrobial spectrum assays. *B. coagulans* MTCC 492 produced maximum amount of bacteriocin at 12 h of incubation. *L. plantarum* MTCC 1407 produced maximum amount of bacteriocin at 20 h of incubation.

Bacteriocin producers *L. plantarum* MTCC 1407 and *B. coagulans* MTCC 492 used in the present study exhibited secondary metabolite kinetics. Generally, bacteriocin production by bacteria is a growth associated process (Leory and De, 2002). It displays secondary metabolite kinetics (Ogunbanwo et al., 2003). Bacteriocin production in the early stationary phase is a characteristic feature of lactic acid bacteria (Tiwari and Srivastava, 2008). Like the lactic acid bacteria (LAB) some representatives of *Bacillus* spp., such as *B. subtilis* and *B. licheniformis*, also produce maximum bacteriocin in stationary phase (Sharp et al., 1992).

The antimicrobial spectrum of the bacteriocins from these two organisms was determined by percentage inhibition method (Table 2). It was chosen over well-diffusion assay which has some disadvantages of medium composition, poor diffusion by bacteriocins, concentration of bacteriocin in the extract, etc. which may result in false negatives. Partially purified bacteriocin of both producers, *L. plantarum* and *B. coagulans* were effective to varying degree against *S. thermophilus* MTCC 1928*, L. mesenteroides* MTCC 107 and *M. flavus* ATCC 10240*.* They also inhibited the growth of Gram negatives such as *E. coli* MTCC 1650 and *P. aeruginosa* ATCC 10602*.*

By and large, *L. plantarum*`s bacteriocin exhibited greater efficacy than bacteriocin produced by *B. coagulans*. Generally, the antibacterial activity of most bacteriocins is directed against species that are closely related to the producer and also against a number of other less closely related bacteria including spoilage bacteria (Schillinger et al., 1991; Ennahar et al., 1996; Rekhif et al., 1995).

A number of studies are being conducted to enhance the antimicrobial spectrum of the bacteriocins for greater application in food systems. Use of more than one bacteriocin is one such approach. The two bacteriocin preparations prepared in the present study were mixed in equal volume and assay performed (Table 2). Combination of these bacteriocins in equal ratio did not result in any appreciable increase in antibacterial activity in this investigation. However, no trend could be established.

Mainly synergistic effects have been reported between pairs of bacteriocins from lactic acid bacteria. Mulet-Powel et al. (1998) reported synergism and were the first ones to report antagonism with combination of bacteriocins. Their study dealt with different bacteriocins (nisin produced by *Lactococcus lactis*, pediocin AcH produced by *Pediococcus acidilactici*, lacticin 481 produced by *L. lactis*, lactacin F produced by *Lactobacillus johnsonii* and lactacin B produced by *Lactobacillus acidophilus*) against 10 different indicator strains. They could not assign any reason for antagonism of different pairs of bacteriocins. Increased antibacterial activity of combination than when used alone has been reported by Hanlin et al. (1993). Bacteriocins were obtained from four producers- *L. lactis*,

Table 2. Percentage inhibition of indicator organisms upon use of bacteriocins of *L. plantarum* MTCC 1407 and *B. coagulans* MTCC 492 independently and in combination.

Indicator organism	Inhibition by bacteriocins of *Lactobacillus plantarum* MTCC 1407 (%)	INHIBITION by bacteriocins of *Bacillus coagulans* MTCC 492 (%)	Inhibition using a combination of bacteriocins (%) (1:1 v/v)
Streptococcus thermophilus MTCC 1928	50	39	53
Micrococcus flavus ATCC 10240	36	32	39
Leuconostoc mesenteroides MTCC 107	65	32	67
Pseudomonas aeruginosa ATCC 10662	63	26	66
Escherichia coli MTCC 1650	72	51	74

P. acidilactici, *Lactobacillus sake* and *Leuconostoc carnosum.* Indicators strains used were *Lactobacillus plantarum* NCDO 955, *L. mesenteroides* Ly, *P. acidilactici* LB-42, *E. faecalis* MB1 and *L. monocytogenes* strains CA and Scott A. Synergism has also been observed by Vignolo et al. (2000). The antilisterial efficiency of three bacteriocins from lactic acid bacteria, lactocin 705 (produced by *L. casei* CRL705), enterocin CRL35 (produced by *E. faecium* CRL35), and nisin, was tested in the broth, individually and in combination against *L. monocytogenes* and *Listeria innocua*.

Antimicrobial action of bacteriocins occurs in steps-adsorption of the bacteriocin on cell wall, its transport across the cell membrane and finally its action within the cytoplasm. Bacteriocins are cationic proteins and their primary receptors are anionic lipids (O`Sullivan et al., 2002). Presence of receptors on cell surface plays a role in bacteriocin specificity (Drider et al., 2006). Synergistic effect occurs when receptor for one bacteriocin is not present but receptor for another bacteriocin is available for antibacterial action. Antagonism can occur when the bacteriocin producers compete for the same receptors on indicator cell surface.

Bacteriocins which belong to different categories and with different mode of actions are likely to exhibit synergistic effect (Vignolo et al., 2000). They suggested the use of combination of bacteriocins belonging to different classes to obtain enhanced activity. Both the bacteriocins used in this study belong to same class. As per the classification proposed by Abriouel et al. (2011), bacteriocins from *Bacillus coagulans* have been classified as belonging to class IIa which are non-modified pediocin like bacteriocins with antimicrobial activity against *Leuconostoc, Oenococcus, Listeria, Pediococcus* and *Enterococcus.* Bacteriocins from *L. plantarum* are generally classified as class IIb. They are small, ≤10 KDa, heat stable, non-lanthinone containing peptides (Chen and Hoover, 2003).

Further work on understanding of mechanism of interaction of bacteriocins is in progress. Use of combination of other bacteriocins from same class and different classes is being pursued. Amino acid sequencing of highly purified form of the bacteriocins can give a greater insight into the nature of interaction with each other.

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Full Length Research Paper

Endophytic fungi from medicinal herb *Salvia miltiorrhiza* Bunge and their antimicrobial activity

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A total of 57 endophytic fungal isolates were obtained from the roots of *Salvia miltiorrhiza* Bunge (Lamiaceae). Fourteen (14) distinct isolates were selected for further taxonomical identification by morphological traits and internal transcribed spacer (ITS) rRNA gene sequence analysis. Twelve (12) genera were identified among which *Alternaria* and *Fusarium* were dominants. Eight endophytic fungi (that is, *Pleosporales* sp. Samif02, *Leptosphaeria* sp. Samif03, *Peyronellaea glomerata* Samif04, *Xylomelasma* sp. Samif07, *Bionectria ochroleuca* Samif08, *Sarocladium kiliense* Samif11, *Petriella setifera* Samif13 and *Cadophora* sp. Samif14) were separated as the endophytic fungi from *S. miltiorrhiza* for the first time. Most of the fungal isolates were observed to have antibacterial activity that suggests antibacterial compounds mainly exist in mycelia. The ethyl acetate extracts of *Alternaria* sp. Samif01, *Xylomelasma* sp. Samif07, *Fusarium redolens* Samif09, *Sarocladium kiliense* Samif11 and *Petriella setifera* Samif13 were also observed to have antifungal activity. Among the isolates, *Alternaria* sp. Samif01 and *Sarocladium kiliense* Samif11 were found to have strong antibacterial and antifungal activities. The results indicate that there is a diversity of the endophytic fungi from *S. miltiorrhiza*, and these endophytic fungi could be an excellent resource for searching natural antimicrobial compounds.

Key words: *Salvia miltiorrhiza* Bunge, endophytic fungi, ethyl acetate extracts, antimicrobial activity, antimicrobial compounds.

INTRODUCTION

Endophytic fungi, colonizing inside the normal plant tissues, are rich and potential resources for producing bioactive metabolites such as antimicrobial, insecticidal, anti-viral, anti-tumor and antioxidant compounds (Strobel, 2003; Kharwar et al., 2011; Chowdhary et al., 2012). Some endophytic fungi have the ability to produce the same or similar bioactive compounds as those that originated from their host plants (Zhao et al., 2011). Isolation of the endophytic fungi which produce certain bioactive substances has also become an efficient method to screen broad-spectrum, stable and low phytotoxic biocontrol agents (Gimenez et al., 2007).

Salvia miltiorrhiza Bunge (Lamiaceae) an important and well-known medicinal herb in Asian countries, commonly known as "Danshen" or "Tanshen" in Chinese, has been widely used as a traditional Chinese medicine (TCM) for treatment of coronary artery diseases, angina pectoris, myocardial infarction, cerebrovascular diseases, various types of hepatitis, chronic renal failure, and menstrual disorders (Wang, 2010; Wu et al., 2012).

Some endophytic fungi have been isolated from species of the genus *Salvia*. Two cytotoxic alkaloids cochliodinol and isocochliodinol were isolated from endophytic fungus *Chaetomium* sp. derived from *S. officinalis* growing in Morocco (Debbab et al., 2009).

Some endophytic fungi from *S. miltiorrhiza* were

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examined to have tanshinone IIA with TLC and HPLC (Wei et al., 2010). The endophytic fungus *Trichoderma atroviride* D16 from the roots of *S. miltiorrhiza* was screened with High-performance liquid chromatography (HPLC) and LC-HRMS/MS to contain tanshinones I and IIA though these tanshinone-producing endophytic fungi should be further verified (Ming et al., 2012).

To the best of our knowledge, there were no reports about the screening of antimicrobial activity on the endophytic fungi from *S. miltiorrhiza*. The aim of this study was to further isolate and identify the endophytic fungi from the roots of *S. miltiorrhiza* as well as to examine the antimicrobial activity of the ethyl acetate extracts on pathogenic bacteria and fungi in order to provide additional data for utilization of the antimicrobial metabolites and these fungi as biocontrol agents.

MATERIALS AND METHODS

Plant materials and isolation of endophytic fungi

The three-year old healthy roots of *S. miltiorrhiza* Bunge were collected from the Institute of Medicinal Plant Development (116°16'27" E, 40°1'59" N), Chinese Academy of Medical Sciences, Beijing, China, in July 2011. The plant was identified according to the morphological features by Prof. Yuhai Guo, a botanist from the College of Agronomy and Biotechnology, China Agricultural University. The voucher specimen (BSMPMI-201107001) of this plant was deposited in the Herbarium of the Institute of Chinese Medicinal Materials, China Agricultural University. The plant samples were stored in the sealed plastic bags at 4°C for processing within 24 h of collection. The isolation of endophytic fungi was performed according to the previous reports with some modifications (Li et al., 2008; Xu et al., 2008).

The root samples were rinsed thoroughly with tap water to remove soil residues and dust, sterilized successively with 75% ethanol for 2 min and immersed in 0.2% mercuric chloride for 20 min, then rinsed in sterile distilled water for four times. After surface sterilization, both root epidermis and remnant tissues were cut into small pieces of 0.5 cm × 0.5 cm respectively, placed on potato dextrose agar (PDA) plates containing 500 µg/mL of streptomycin sulfate and incubated at 25°C until mycelia were apparent on PDA plates. Pure cultures were finally isolated by hyphal tip isolation on PDA plates without antibiotics and stored at 4°C.

Morphological characterization

The isolated fungi were observed and described according to the methods of Ainsworth et al. (1973), Photita et al. (2005), and Li et al. (2008), including colony morphology and microscopic observation of mycelia and asexual/sexual spores.

Colonization frequency of fungal endophytes

The colonization frequency (CF, %) of endophytes was calculated according to the method of Hata and Futai (1995):

$$CF\ (\%) = (N_{COL}/N_t) \times 100$$

Where, N_{COL} is the number of segments colonized by each fungus and N_t is the total number of segments.

DNA extraction, ITS-rDNA amplification and sequence analysis

Endophytic fungi were also identified based on the analysis of the ITS sequences of rDNA regions. Total genomic DNA of the fungal isolates was extracted according to the protocols described by Wang et al. (1993) and Jasalavich et al. (2000). The ITS regions were amplified by the polymerase chain reaction (PCR) with the primer pair ITS1 (5'- TCCGTAGGTGAACCTGCGG -3') and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Li et al., 2008; Xu et al., 2008; Zhong et al., 2011). For identification, the PCR products were purified using the QIA quick gel purification kit (Qiagen, Hilden, Germany) as described by the manufacturer's protocol and sequenced using the primer pair ITS1 and ITS4 on the ABI PRISM 3730 sequencer (Applied Biosystem, USA).

The sequences of the endophytic fungal strains were run by BLASTN program against the database (National Center for Biotechnology Information website: http://www.ncbi.nlm.nih.gov), and then they were submitted to GenBank database where the accession numbers were obtained.

The sequences were aligned using the CLUSTAL×2.0 program and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0. The Kimura two-parameter model was used to estimate evolutionary distance. The phylogenetic reconstruction was done by using the neighbor-joining (NJ) algorithm (Naruya and Masatoshi, 1987), with bootstrap values calculated from 1,000 replicate runs, using the software routines included in the MEGA software.

Mycelia suspension culture and ethyl acetate extract preparation

Three mycelia plugs from the edge of the actively growing colony were inoculated into 500 mL Erlenmeyer flasks containing 200 mL potato dextrose broth (PDB). The cultures were incubated at 150 rpm on a rotary shaker at 25°C for 20 days. After suspension culture, the fermented broth was filtrated under vacuum to afford the filtrate and mycelia. The filtrates were extracted thrice with an equal volume of ethyl acetate (1:1, v/v). The mycelia were dried and powdered, followed by extraction with ultrasound in ethyl acetate for three times. The ethyl acetate extracts from the mycelia and filtrate were obtained by evaporation under vacuum, respectively.

Detection of antimicrobial activity of the ethyl acetate extracts

The antimicrobial activities of the ethyl acetate extracts were detected by TLC-bioautography assay (Zhao et al., 2008). Four bacterial strains including two Gram-positive (*Bacillus subtilis* ATCC 11562 and *Clavibacter michiganensis* LP-0301) and two Gram-negative (*Agrobacterium tumefaciens* ATCC11158 and *Pseudomonas lachrymans* ATCC11921) bacteria were selected for antibacterial assay. The TLC plate covered with the test bacterium was incubated at 28°C for 12 h, then sprayed with 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, purchased from Amresco, USA), and incubated successively for another 10 min. The antibacterial activity of the ethyl acetate extracts was determined by the formation of well-defined inhibition zones made visible by spraying with MTT that was converted to a formazan dye by the living microorganism (Bernas and Dobrucki, 2000). Antibacterial activity was detected as the white inhibition zones against a purple background, and the length of each antibacterial area was also measured in order to calculate its R_f value:

$$R_f = D_1/D_2,$$

Where, D_1 is the distance (mm) between the antimicrobial area and

Table 1. Colonization frequency (CF) of the endophytic fungi, their closest relatives based on the data from BLAST analysis and morphological identification.

Fungal isolate	CF (%)	GenBank accession number	Closest related species	Similarity (%)	Macro- and microscopic identification
Samif01	14.52	KC878695	*Alternaria* sp. KC139492	100	*Alternaria* sp.
Samif02	4.84	KC878696	*Pleosporales* sp. JN859326	99	*Pleosporales* sp.
Samif03	4.84	KC878697	*Leptosphaeria* sp. GU934537	99	*Leptosphaeria* sp.
Samif04	3.22	KC878698	*Peyronellaea glomerata* KC33977	99	*Peyronellaea* sp.
Samif05	3.22	KC878699	*Phoma pedeiae* GU237770	99	*Phoma* sp.
Samif06	4.84	KC878700	*Phoma eupyrena* HQ115670	100	*Phoma* sp.
Samif07	8.06	KC878701	*Xylomelasma* sp. FR837913	99	*Xylomelasma* sp.
Samif08	6.45	KC878702	*Bionectria ochroleuca* JQ794833	100	*Bionectria* sp.
Samif09	6.45	KC878703	*Fusarium redolens* HQ443207	100	*Fusarium* sp.
Samif10	9.68	KC878704	*Fusarium* sp. JX243851	100	*Fusarium* sp.
Samif11	4.84	KC878705	*Sarocladium kiliense* JX499275	100	*Sarocladium* sp.
Samif12	6.45	KC878706	*Aspergillus* sp. JX029073	100	*Aspergillus* sp.
Samif13	8.06	KC878707	*Petriella setifera* JX501314	100	*Petriella* sp.
Samif14	6.45	KC878708	*Cadophora* sp. JN859262	96	*Cadophora* sp.

initial sample point, and D_2 is the distance (mm) between the developing solvent front and initial sample point on a TLC plate (Zhong et al., 2011).

Two phytopathogenic fungi *Fusarium oxysporum* f.sp. *niveum* and *Magnaporthe oryzae* were also selected for antifungal assay. The TLC plate was spread with the test fungal conidia. Then, it was incubated at 25°C for 4 to 7 days; the inhibition zone of mycelia growth was visible, and the R_f value of the antifungal area was determined without MTT treatment. All the test bacteria and fungi were provided by the Department of Plant Pathology, China Agricultural University, and TLC-bioautography assay was performed in three times.

RESULTS AND DISCUSSION

Identification of the endophytic fungi

A total of 57 endophytic fungal isolates were separated from the root epidermis and remnant tissues of *S. miltiorrhiza*. According to their morphological characters (the shape of conidia, type of conidiophores, growth rate, colony color and texture), 14 representative fungal isolates were selected for further macro and microscopic identification. They were identified as 12 genera including *Alternaria* (Samif01), *Pleosporales* (Samif02), *Leptosphaeria* (Samif03), *Peyronellaea* (Samif04), *Phoma* (Samif05 and Samif06), *Xylomelasma* (Samif07), *Bionectria* (Samif08), *Fusarium* (Samif09 and Samif10), *Sarocladium* (Samif11), *Aspergillus* (Samif12), *Petriella* (Samif13) and *Cadophora* (Samif14) (Table 1). Among them, *Alternaria* (Samif01) and *Fusarium* (Samif09 and Samif10) were two dominant genera with their colonization frequency (CF) as 14.52% and 16.13%, respectively.

The ITS1-5.8S-ITS4 partial sequences of 14 distinct isolates were submitted to the GenBank to obtain their accession numbers (i.e. KC878695 - KC878708), and the closest related species were got by BLAST analysis (Table 1). Except for Samif14, other isolated endophytic fungi had homology greater than or equal to 99% to their closest related species. Fourteen (14) isolates were identified on the basis of morphological traits and ITS rRNA gene sequence analysis. The molecular characters of the endophytic fungi were basically coincident with their morphology. For example, isolate Samif03 had a dense colony with white to grey mycelia, and spherical shaped conidia. It was tentatively identified as *Leptosphaeria* sp. (Camara et al., 2002). The closest sequence similarity of isolate Samif03 was 99% to the fungus *Leptosphaeria* sp. (GU934537) in GenBank. In agreement with the morphology-based diagnosis, isolate Samif03 was clustered in a clade containing *Leptosphaeria* sp. (GU934537) with 100% NJ bootstrap support (Figure 1). On the basis of the ITS sequence and morphological traits, isolate Samif03 was considered as the member of the genus *Leptosphaeria*, and identified as *Leptosphaeria* sp. (Ainsworth et al., 1973; Camara et al., 2002).

Comparison of the ITS-rDNA sequences obtained from

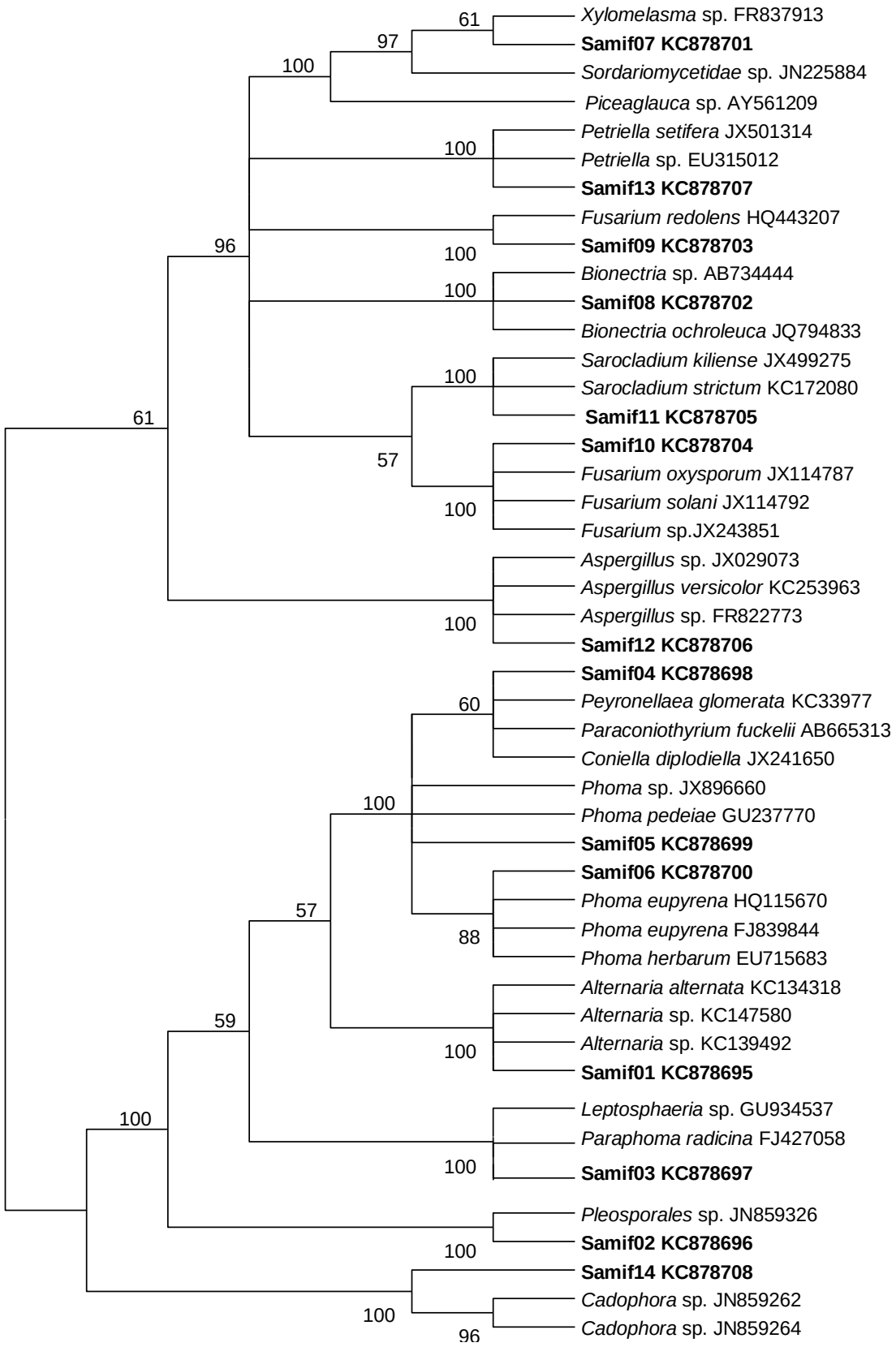

Figure 1. Phylogenetic relationship analysis of the fungal isolates Samif01 to Samif14 from *S. miltiorrhiza* Bunge to other fungi from GenBank, deduced from the ITS rDNA sequences. The numbers at the branches indicate the percentages of trees from 1000 bootstrap replication in which the branch occurs. The unrooted tree was generated using Clustal×2.0 program by Neighbor-Joining method. Phylogeny test was computed by MEGA 4.0.

Table 2. Antibacterial activity of the ethyl acetate extracts from the endophytic fungi by TLC-bioautography-MTT assay.

Fungal isolate	M/F	R_f value of the antibacterial area (Diameter of the antibacterial area)			
		Agrobacterium tumefaciens	*Bacillus subtilis*	*Clavibacter michiganensis*	*Pseudomonas lachrymans*
Samif01	M	0-0.57 (+++)	0-0.53 (+++)	0-0.53 (+++)	0-0.45 (+++)
	F	0-0.55 (+++)	0-0.52 (+++)	0-0.52 (+++)	0-0.42 (+++)
Samif02	M	0-0.47 (++)	0-0.18(++), 0.35-0.68(+)	0.63-0.68 (+)	0-0.43 (++)
	F	0-0.13 (+)	0-0.57(++), 0.65-0.72(+)	0-0.18(++), 0.37-0.70(+)	0-0.10 (+)
Samif03	M	0-0.33(++), 0.45-0.58(+)	0-0.53 (++)	0-0.52 (++)	0-0.35 (++)
	F	0-0.20 (+)	0-0.32 (++)	0-0.30 (+)	0-0.18 (+)
Samif04	M	0-0.38 (++)	0-0.37 (++)	0-0.35 (++)	0-0.42 (++)
	F	nd	nd	nd	nd
Samif05	M	0-0.45 (++)	0-0.42 (++)	0-0.35 (++)	0-0.30 (+)
	F	0-0.03 (+)	0-0.22 (++)	0-0.20 (++)	nd
Samif06	M	0-0.35 (+)	0-0.28 (++)	0-0.27 (++)	0-0.32 (+)
	F	nd	0-0.18 (+)	0-0.18 (+)	nd
Samif07	M	0-0.15 (+)	0-0.15 (+)	0-0.15 (+)	0-0.23 (+)
	F	0-0.03 (+)	0-0.23 (+), 0.35-0.43 (+)	0-0.30 (+), 0.35-0.47(+)	0-0.17 (+)
Samif08	M	0-0.30 (++)	0-0.37 (++)	0-0.35 (++)	0-0.30 (++)
	F	0-0.45 (+)	0-0.72 (++)	0-0.70 (++)	0-0.35 (+)
Samif09	M	0-0.53 (++)	0-0.52(++), 0.57-0.65(+)	0-0.53(++), 0.57-0.67(+)	0-0.72 (++)
	F	0-0.20 (++)	0-0.27 (++)	0-0.28 (+)	0-0.38 (+)
Samif10	M	0-0.43 (++)	0-0.51 (++)	0-0.57 (++)	0-0.60 (++)
	F	0-0.18 (++)	0-0.27 (++)	0-0.28 (+)	0-0.36 (+)
Samif11	M	0-0.72 (++)	0-0.43(++),0.57-0.68(+)	0-0.68 (++)	0-0.72 (++)
	F	0-0.42 (++)	0-0.32 (++)	0-0.32 (++)	0-0.48 (++)
Samif12	M	0-0.02 (+)	0-0.05 (+)	Nd	Nd
	F	0-0.03 (+)	0-0.17 (+)	0-0.23 (+)	Nd
Samif13	M	0-0.40 (+)	0-0.42 (++)	0-0.38 (++)	0-0.42 (+)
	F	0-0.05 (+)	0-0.20 (+)	0-0.18 (+)	Nd
Samif14	M	0-0.23 (+)	0-0.63 (++)	0-0.68 (+++)	0-0.20 (+)
	F	0-0.73 (+)	0-0.18 (+)	nd	0-0.68 (+)

M, mycelia ethyl acetate extract; F, filtrate ethyl acetate extract; developing solvent system in TLC was petroleum ether-acetone (2:1, v/v); nd, antimicrobial activity was not detected; +, the diameter of the antimicrobial activity area was 0-5 mm; ++, the diameter of the antimicrobial activity area was 5-10 mm; +++, the diameter of the antimicrobial activity area was more than 10 mm; The positive control was streptomycin sulfate which was only sampled on the TLC plate and showed antibacterial activity.

the isolates with the sequences available in the GenBank databases allowed us to analyze the phylogenic affiliation of these fungi (Figure 1). The phylogenic relationship demonstrated that the isolates could be sorted to two groups (clades). The first group was composed of isolates Samif07, Samif08, Samif09, Samif10, Samif11, Samif12 and Samif13.

The second group was composed of isolates Samif01, Samif02, Samif03, Samif04, Samif05, Samif06 and Samif14. To the best of our knowledge, Samif02 (*Pleosporales* sp.), Samif03 (*Leptosphaeria* sp.), Samif04 (*Peyronellaea glomerata*), Samif07 (*Xylomelasma* sp.), Samif08 (*Bionectria ochroleuca*), Samif11 (*Sarocladium kiliense*), Samif13 (*Petriella setifera*) and Samif14 (*Cadophora* sp.) were isolated from *S. miltiorrhiza* Bunge for the first time.

Detection of antimicrobial activity

Tables 2 and 3 show the antimicrobial activity of the ethyl acetate extracts obtained from the isolated fungi by using TLC-bioautography assay. R_f values of the antimicrobial areas can indicate the relative polarity of the active compounds in the samples, and the diameters can indicate the relative antimicrobial activity of the compounds (Bernas and Dobrucki, 2000; Zhong et al., 2011). Most of the ethyl acetate extracts except the filtrate extract of isolate Samif04 showed antibacterial activity to some extent, and antibacterial compounds mainly existed in mycelia. The ethyl acetate extracts of isolates Samif01, Samif08, Samif09, Samif10 and Samif11 were found to have stronger antibacterial activity than other fungal extracts. Compared with antibacterial

Table 3. Antifungal activity of the ethyl acetate extracts from the endophytic fungi on plant fungal pathogens by TLC-bioautography assay.

Fungal isolate	M/F	R_f value of the antifungal area (Diameter of the antifungal area)	
		Fusarium oxysporum f. sp. *niveum*	*Magnaporthe oryzae*
Samif01	M	0-0.18 (++)	0-0.40 (+++)
	F	0-0.20 (++)	0-0.43 (+++)
Samif02	M	nd	0.63-0.67 (+)
	F	0.53-0.60 (+)	0.52-0.61 (+)
Samif03	M	nd	0-0.10 (+)
	F	nd	0-0.08 (+)
Samif04	M	nd	nd
	F	nd	nd
Samif05	M	nd	nd
	F	nd	nd
Samif06	M	nd	0-0.06 (+)
	F	nd	nd
Samif07	M	0-0.10 (+)	0-0.13 (+)
	F	0-0.10 (+)	0-0.13 (+)
Samif08	M	0-0.12 (+)	nd
	F	nd	nd
Samif09	M	0-0.20 (+)	0-0.03 (+)
	F	0-0.15 (+)	0-0.10 (+)
Samif10	M	nd	0-0.21 (+)
	F	nd	0-0.03 (+)
Samif11	M	0-0.10 (++)	0-0.27 (++)
	F	0-0.03 (++)	0-0.11 (++), 0.35-0.51 (+)
Samif12	M	nd	0-0.06 (+)
	F	nd	0-0.10 (+)
Samif13	M	0-0.67 (+)	0-0.52 (+)
	F	0-0.03 (+)	0-0.08 (+)
Samif14	M	0-0.05 (+)	0-0.05 (+), 0.30-0.40 (+)
	F	nd	0-0.08 (+), 0.34-0.41 (+)

The positive control was carbendazim which was only sampled on the TLC plate and showed antifungal activity. Other notes are the same as those in Table 2.

activity, only a few endophytic fungal extracts (isolates Samif01, Samif07, Samif09, Samif11 and Samif13) showed antifungal activity on two test phytopathogenic fungi (Table 3). The ethyl acetate extracts of isolates Samif01 and Samif11 were also found to have stronger antifungal activity than other extracts. The results indicate that both antifungal and antibacterial compounds mainly exist in the ethyl acetate extracts of isolates Samif01 and Samif11.

Conclusion

In this study, we reported the endophytic fungi from the roots of *S. miltiorrhiza* Bunge and the detection of the antimicrobial activities of their ethyl acetate extracts. Twelve (12) genera were identified among which both *Alternaria* and *Fusarium* were dominant endophytes. Fourteen (14) fungal isolates were identified by both morphological and molecular methods. Eight endophytic fungi such as *Pleosporales* sp. Samif02, *Leptosphaeria* sp. Samif03, *Peyronellaea glomerata* Samif04, *Xylomelasma* sp. Samif07, *Bionectria ochroleuca* Samif08, *Sarocladium kiliense* Samif11, *Petriella setifera* Samif13 and *Cadophora* sp. Samif14 were separated as the endophytic fungi from *S. mitlorrhiza* for the first time. Some fungal isolates (*Alternaria* sp. Samif01 and *Sarocladium kiliense* Samif11) displayed strong anti-bacterial and antifungal activities. The results indicate that there is a diversity of the endophytic fungi from *S. miltiorrhiza*, and these endophytic fungi will have a great potential as producers of natural antimicrobial compounds.

Further investigation will focus on the isolation of the antimicrobial compounds from these fungi as well as on their applications as biocontrol agents. Other biological activities (cytotoxic, insecticidal, and antioxidant activities) of the endophytic fungi from *S. miltiorrhiza* also should be studied in detail.

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Full Length Research Paper

Detection of *ica*A and *icaD* genes and biofilmformation in *Staphylococcus* spp. isolated from urinary catheters at the University Hospital of Tlemcen (Algeria)

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***Staphylococcus* spp. is a major cause of infections associated with urinary catheterization and other medical devices. Biofilm formation is an important step in the pathogenesis of these *Staphylococci* and depends on the expression of the *ica*ADBC operon involved in the synthesis of a polysaccharide intercellular adhesion. In this study, forty-four (44) *Staphylococcus* spp. obtained from urinary catheters at the University Hospital of Tlemcen (North-West Algeria) were analyzed to investigate the presence or absence of the intercellular adhesion *ica*A and *ica*D genes, using the polymerase chain reaction (PCR). Phenotypic biofilm formation was examined by tissue culture plate (TCP) and Congo red agar (CRA) methods. Seventeen (17) of 44 isolates were shown to carry ica-specific DNA, 18 produced slime on CRA plates but only eight produced biofilm spontaneously on the polystyrene surfaces, under normal growth conditions. Upon induction by sugar, biofilm formation could be stimulated in seven (7) of nine (9)*ica* positive. Biofilm-negative isolates indicated that the *ica*ADBC expression was down-regulated in these strains. Staphylococcus strains isolated from urinary catheters showed high levels of resistance to penicillin (98%) and gentamicine (75%). The data obtained shows the important role of *ica*-genes, the phenotypic variability of biofilm formation and the multi-resistance to antibiotics as virulence factors of *staphylococcus* spp. from urinary catheters.It confirms the complexity and diversity of regulation mechanisms implicated in biofilm formation.**

Key words: *Staphylococcus* spp., urinary catheter, Biofilm, ica operon, slime, tissue culture plate (TCP).

INTRODUCTION

Staphylococcus spp, commensal microorganisms routinely found on the human skin and in the hospital environment (Chokr et al., 2007), are most often associated with chronic infections related to implanted medical devices (Espinasse et al., 2010).

Urinary tract infections can also be caused by these organisms and occur preferentially in patients carrying indwelling urinary tract catheters (Singhai et al., 2012). Urinary catheters have become the second most frequently used medical devices inserted into the human body. Over 40% of nosocomial infections involve the urinary tract, especially in catheterized patients (Holá et al., 2010).

Certain *Staphylococcus* spp. strains are able to form

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biofilms on polymer surfaces and it is suggested that this property contributes significantly to the pathogenesis of staphylococcal infection (Cho et al., 2002). Biofilms are a population of multilayered cells growing on a surface and enclosed in the exopolysaccharide matrix. (Cafiso et al., 2004)

The development of a biofilm is considered to be a two-step process. First, the bacteria adhere to a surface mediated by a capsular antigen, namely the capsular polysaccharide/adhesin (PS/A), then the bacteria multiply to form a multilayered biofilm, with production of polysaccharide intercellular adhesin (PIA) which mediates cell to cell adhesion (Nasr et al., 2013; EL Farran et al., 2013) and provides the protection against opsonophagocytosis and antimicrobial peptideactivity (Spiliopoulou et al., 2012)

The synthesis of PIA is encoded by the products of the chromosomal *ica*-genes (intercellular adhesion), which are organized in an operon structure. The operon contains the *ica* ADBC genes, in addition to the *ica* Rgene which exerts a regulatory function and is transcribed in the opposite direction. Once this operon is activated, four proteins are transcribed, *Ica*A, *Ica*D, *Ica*B and *Ica*C, which are necessary for the synthesis of PIA (Cafiso et al., 2004; Atshan et al., 2012; Agarwal and jain, 2013; Mertens and Ghebremedhin, 2013). PIA is synthesized from UDP-N-acetylglucosamine by N-acetylglucosaminyl-transferase which is encoded by the *ica* locus, particularly *ica*A.

The expression of this gene alone induces low enzymatic activity and production of low amount of polysaccharide. However, the simultaneous expressions of *ica*A and *ica*D promote a significant increase in N-acetylglucosaminyltransferase, with a con-sequent increase in the amount of polysaccharide, hence forming oligomers of 10-20 b-1,6-Nacetylglucosamine residues (Dobinski et al., 2002; Gotz, 2002; Oliveira et al., 2010 ; Gad et al., 2012; Namvar et al., 2013). *Ica*B is the deacetylase responsible for the deacetylation of mature PIA. In addition, the transmembrane protein *Ica*C seems to be involved in the externalization and elon-gation of the growing polysaccharide (Diemond-Hernández et al., 2010).

The expression of the *ica* operon, and as a result the formation of biofilms, seems to be highly variable among staphylococci. Thus, biofilm formation is influenced by the environmental signals and can be induced in response to external stress and subinhibitory concentrations of certain antibiotics (Ziebuhr et al., 1997; Mempel et al., 1994; Cho et al., 2002; Mertens and Ghebremedhin, 2013).

The differentiation of staphylococci with respect to their biofilm phenotype might help to elucidate the impact of staphylococci for the diagnosis of infections associated with biomedical devices. These observations can be useful in the prevention of device-related infections (Mathur et al., 2006).

Several studies have been published on the detection of the *Ica* gene among the staphylococci strains (Cho et al., 2002; Chaieb et al., 2005; Touati et al., 2007; Gad et al., 2009; Wang et al., 2010; Duran et al., 2010; Nasr et al., 2013). However, despite the increasing interest in the subject in recent years, data collection from medical institutions in Algeria are relatively difficult, hence the low number of related studies.

The objective of the present study was to characterize *Staphylococcus* spp*.* strains isolated from urinary catheter at the University Hospital of Tlemcen, in terms of their antibiotic susceptibility, biofilm formation and presence of *ica*A and *ica*D genes.

MATERIALS AND METHODS

Bacterial strains

The strains used in this study were isolated from urethral catheterization from the Intensive Care Unit, Urology and Neurology Services at the University Hospital Center (CHU) of Tlemcen (North-West Algeria). Urinary catheters were removed aseptically from patients suffering from a catheter-related urinary tract infection. The urinary catheters under study are Latex probes not impregnated with antibiotics, transported at 4°C, and immediately analyzed at the laboratory.

Identification

After removal of the catheter, the microbiological analysis was performed using the Brun-Buisson technique (Brun-Buisson, 1994), which consists in rinsing the catheter lumen with saline water and vortexing its intravesical end before cultivation on Chapmanagar medium which allows the selection of staphylococci.

Moreover, all isolates were identified by classic microbiological methods including colony morphology, Gram staining, catalase test, coagulase test and the Api-Staph test (BioMérieux®).

Antibiotic sensitivity test

Antimicrobial susceptibility testing was performed in accordance with the guidelines established by the Antibiogram Committee of the French Microbiology Society (Soussy, 2010) using 17 antibiotic discs including: Penicilin (10 ug), Oxacillin (5 ug),Cefoxitin (30 ug), Gentamicin (10 ug), Tobramycin (10 ug), Amikacine (30 ug), Vancomycine (30 ug), Rifampim (30 ug), Fosfomycin (50 ug), Fusidic Acid (10 ug), Clindamycin (2 ug), Pristinamycin (15 ug), Erythromycin (15 ug), Ofloxacin (5 ug), Tetracycline (30 ug), Chloramphenicol (30 ug) and Trimethoprime/sulfamethoxazole (25 ug).

Detection of biofilm formation

Tissue culture plate method (TCP)

Quantitative determination of biofilm formation on 96-well tissue culture plates (Sigma, UK) was performed based on the Christensen method (Christensen et al., 1985), with a modification in the incubation length which was extended to 48 h. Therefore, the biofilm production was evaluated in three different media, namely

Brain Heart Infusion Broth (BHIB), BHIB with 2% sucrose, and BHIB with 1% glucose.

The bacteria were grown overnight in respective media, and the cultures were then diluted 1:100 and incubated in a microtiter polystyrene plate at 37°C. Microtiter wells were washed three times with distilled water, dried in an inverted position, and stained with 0.5% (w: v) crystal violet solution (Mathur et al., 2006). The adherent cells were resuspended in 95% ethanol solution and the absorbance was measured at 540 nm with a micro ELISA auto reader (model 680, Biorad, UK). The isolates were classified into three categories: a) non adherent, optical density lower than 0.120; b) weakly adherent, optical density greater than 0.120 and smaller or equal to 0.240, c) strongly adherent, optical density greater than 0.240.

Congo red agar method (CRA)

The Congo red test was performed as previously described by Freeman et al. (1989). The medium consisted of brain heart infusion broth (BHIB, 37 g/L), sucrose (50 g/L), agar no.1 (10 g/L) and Congo red stain (0.8 g/L). Congo red was prepared as a concentrated aqueous solution and autoclaved at 121°C for 15 min, separately from the other medium constituents and was then addedto the mixture when the agar had cooled to 55°C. The plates were inoculated and incubated aerobically for 24 to 48 h at 37°C.

Biofilm producers form black colonies on CRA, whereas non-producers form red colonies. The Congo red dye directly interacts with certain polysaccharides, forming colored complexes (Jain and Agarwal, 2009).

Detection of icaA and icaD loci

Extraction of bacterial DNA was performed by thermal shock. After overnight culture on Luria Bertani agar plates (Bio-Rad, Marnes-la-Coquette, France), 5 colonies were suspended in 500 ml of DNase and RNase-free water (Invitrogen, England). The suspension was boiled at 100°C for 10 min in a thermal block (Polystat 5, French), then centrifuged at 15000 rpm for 5 min. An aliquot of 2 µL of the supernatant was used as DNA template for PCR.

The presence of *ica*A and *ica*D DNA was demonstrated by polymerase chain reaction (PCR) using forward and reverse primers for *ica*A and *ica*D. For *ica*A, the forward primer (corresponding to nucleotides 1337-1356) had the following sequence: 5'-TCT CTT GCA GGA GCA ATC AA-3'; and the reverse primer (corresponding to nucleotides 1505-1524) had this sequence: 5'-TCA GGC ACT AAC ATC CAG CA-3'. The primer sequences for *ica*D were: forward (nucleotides 1963-1982), 5'-ATG GTC AAG CCC AGA CAG AG- 3'; and reverse (nucleotides 2138-2160), 5'-CGT GTT TTC AAC ATT TAA TGC AA-3'. PCR amplification was carried out according to the parameters described by Arciola et al. (2001) and visualization of the amplified products by a 2% gel electrophoresis.

RESULTS

Characterization of staphylococcal isolates froma urinary catheter

A total of 44 strains were collected from a urinary catheter used more than 48 h at the University Hospital of Tlemcen. After biochemical analysis, all 44 strains were identified as staphylococcal species and included: 21 *S. epidermidis*, 11 *S. saprophyticus*, 11 *S. aureus*, and 1 *S. hominis*.

Detection of biofilm production

Detection of slime-producing staphylococcus strains

Phenotypic production of slime by all strains under study was assessed by culture on CRA plates.

Among the 44 clinical isolates, 18 (41%) staphylococcus strains were slime producers.These included 6/11 *S. saprophyticus*, 5/11 *S.aureus*, *1/1 S.hominis* and 6/21 *S.epidermidis*. The remaining 26 were non-slime producers.

Study of biofilm production by tissue culture plate (TCP) method

Quantitative determinations of biofilm formation were carried out by measuring the adherence of broth cultures to 96-well tissue culture plates, as outlined in materials and methods. Under standard growth conditions in BHIB, only 8 (18%) out of 44 isolates were capable of forming a biofilm.

To evaluate the impact of environmental growth conditions on biofilm formation by clinical isolates, we performed some biofilm assays using growth media supplemented with 1% glucose or 2% sucrose, as previouslydescribed. This resulted in an increase in the number of isolates capable of biofilm formation; 15 (34%) isolates out of 44 were able to produce a biofilm, in the presence of one of these media supplements. Thus, the overall rate of biofilm-forming strains rose from 18 to 34% after stimulation (Figure 1).

Detection of icaA andica D loci by PCR

The PCR technique was applied to all 44 staphylococcal strains. The *ica*A and *ica*D genes were detected concomitantly in 17 (38.5%) of the 44 staphylococcal isolates. Furthermore, 2 of them presented the loci *ica*Donly.

Relationships between the presence of the ica operon, slime production and the TCP method

Sixteen (16) of the 17 *ica*A and *ica*D positive strains were found to be slime producers and 8 produced a visible biofilm on polystyrene surfaces under standard growth conditions. After stimulation by sugar supplementations, 7 out of 9 of the previous *ica*A/*ica*D positive and biofilm-negative strains formed a visible biofilm on polystyrene tissue culture plates.

However, two *ica*-positive isolates remained biofilm-negative even after exposure to these biofilm-inducing growth conditions. Both strains*ica*A- and*ica*D do not form biofilmbutareslime-producers; however *ica*A- and *ica*D+ neither produced slime nor formed biofilm.

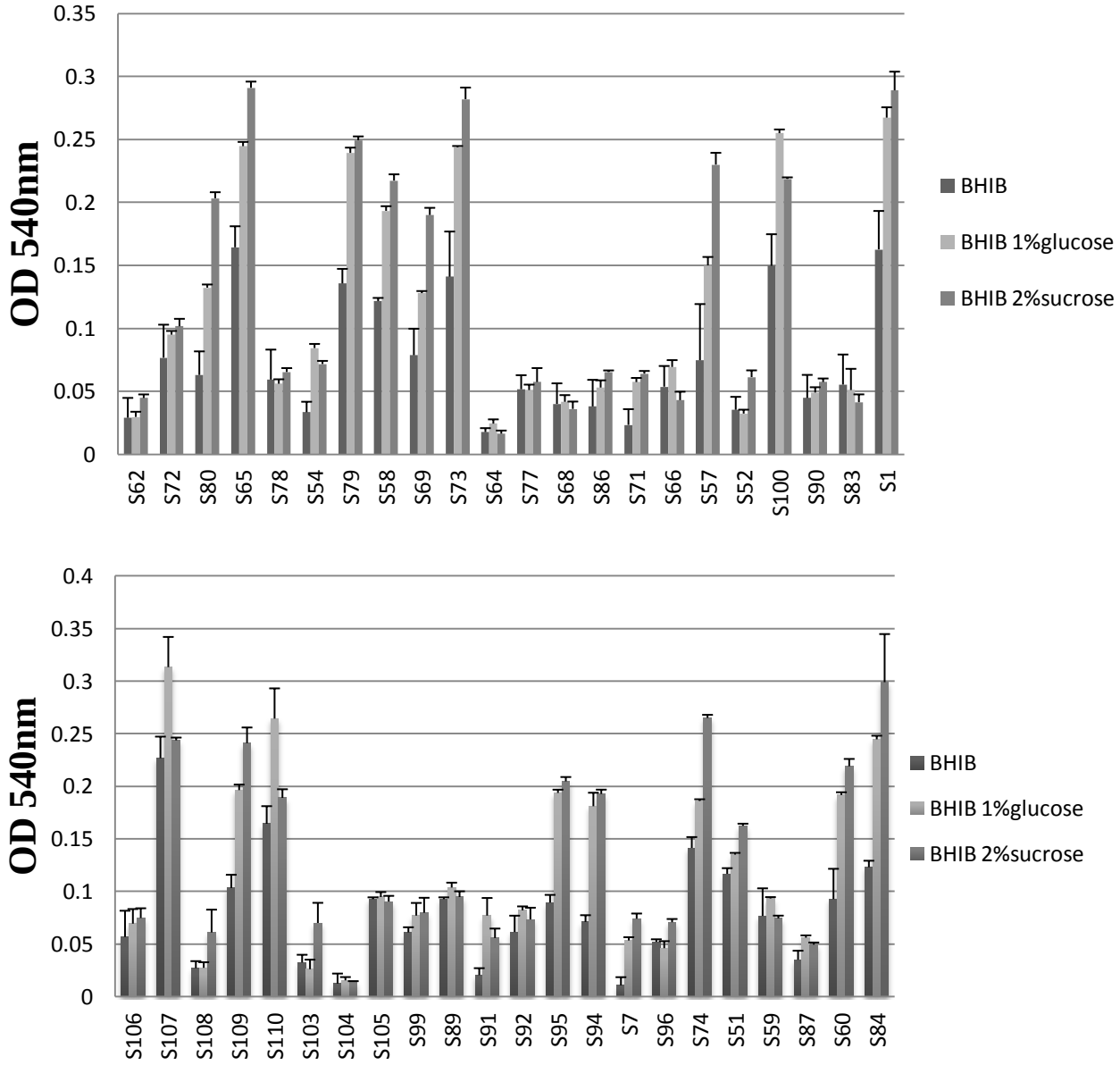

Figure 1. Biofilm formation of Staphylococcus strains on BHIB, BHIB 1%glucose and BHIB 2% sucrose. Adherent bacterial biofilms were stained with Crystal violet as described in Materials and methods. A strain was considered biofilm-positive, if its OD was higher or equal to 0.120, P< 0:05 (t-test). Data are representative of 3 replicate experiments. *S. saprophyticus*: S106, S107, S110, S104, S60, S84, S78, S86, S66, S5 2, S1; *S. aureus*: S105, S89, S62, S80, S65, S54, S79, S77, S57, S100, S83; *S.epidermidis:* S108, S109, S103, S91, S92, S95, S94, S7, S96, S74, S51, S59, S87, S72, S58, S69, S73, S64, S68,S71, S90; *S. hominis:* S99.

All 23 *ica*A/*ica*Dnegative strains were unable to produce slime on CRA and biofilm on polystyrene tissue culture plates. The results obtained with all the strains are summarized in Table 1.

Antibiotic sensitivity testing

Antibiotic susceptibility testing showed that most of *Staphylococcus* spp. strains were resistant to more than nine antibiotics and were found to be susceptible to four major antibiotics: Rifampim, Fosfomycin, Clindamycin and Chloramphenicol. Moreover, no strains were found to be vancomycin- and pristinamyci-resistant.Biofilm-producing strains were found to be more resistant compared to non-producing ones.

The total percentage of resistance against each antibiotic and the relationship between biofilm formation and antimicrobial resistance pattern are represented in Table 2.

Table 1. relationships between the presence of the *ica* operon and biofilm production.

Microorganism	Number of isolates	Production of slime	Biofilm			*icaA*	*icaD*
			BHIB	BHIB 1%glu	BHIB 2%sac		
S. epidermidis	21	6	2	6	6	6	6
S. aureus	11	5	2	4	4	5	7
S. saprophyticus	11	6	4	5	5	5	5
S. hominis	1	1	0	0	0	1	1
Total	44	18	8	15	15	17	19

DISCUSSION

In the last two decades, with the increasing use of indwelling medical devices, nosocomial infections caused by Gram-positive bacteria, in particular *staphylococcus* spp., have become more prevalent as a cause of hospital-acquired infection (Fitzpatrick et al., 2002).

The major pathogenic factor is the ability to produce an extracellular slime and form a biofilm, thus making the clinical treatment extremely difficult. The biofilm development process requires polysaccharidic intercellular adhesin, which is synthesized by the enzymes encoded by the intercellular adhesion cluster (*ica*) (Martín-López et al., 2002).

Early detection and management of biofilm-forming staphylococci can be one of the essential steps towards the prevention and management of device-associated nosocomial infections (Nasr et al., 2013).

In our study, 44 *staphylococcus* spp. were isolated from a urinary catheter in order to test the occurrence of slime genes, biofilm production and slime production in staphylococci using PCR, TCP and Congo red agar methods, respectively.

The results reveal that *S. epidermidis* are the most frequently isolated species, corresponding to 48% of all strains. Other staphylococcus species were also identified, including *S. saprophyticus*, *S. aureus* and *S. hominis*. These results are close to those obtained by Diemond-Hernández et al. (2010).

It has been noticed in several studies that the *S. epidermidis* is the most frequently isolated speciesin nosocomial infections and is the most common causative organism found in infections of implanted medical devices. It makes up a significant part of the normal bacterial flora of the human skin and mucous membranes and is probably easily introduced as a contaminant during the surgical implantation of the polymeric device (Otto, 2008).

In this study, *ica*A and *ica*D were detected concomitantly in 17 of the 44 *staphylococcus* spp. strains isolated from a urinary catheter and an *icaA-/icaD*+ profile was found in two strains.

These results are close to those obtained by Cafiso et al. (2004) who investigated the presence of genes involved in biofilm production and found that 35% of isolates were positive for the *ica*A and *ica*D genes and some of them carried the *ica*D gene only.

In the TCP assay, with BHIB used as standard growth medium, 8 of 17 *ica*A/D positive strains exhibited a biofilm. This is in agreement with the observations of other investigators (Cho et al., 2002; Mathur et al., 2006; Johannes et al., 2002) who found that few or no biofilm-producing isolates could be detected using this medium. Surprisingly, supplementation of BHIB medium with different sugars (BHIB $_{2\%suc}$, BHIB $_{1\%glu}$) increased biofilm formation, and 34% of the studied isolates formed a biofilm in at least one of the used media. Furthermore, two isolates of staphylococci *ica* D+/*ica* A- did not form a biofilm in both media.

These observations suggested that biofilm formation in *staphylococcus* spp is strongly dependent on growth conditions, and indicated that the use of various sugar supplementations is essential for biofilm formation (Mathur et al., 2006).

Moreover, the expression of the *ica* operon and therefore the formation of biofilms seems to be highly variable among staphylococci (Ziebuhr et al., 1997; Mempel et al., 1994). Thus, the biofilm expression is influenced by environmental signals and can be induced in response to external stress and subinhibitory concentrations of certain antibiotics (Cho et al., 2002). Cramton et al. (2001) suggested that anaerobiosis strongly increases biofilm expression. The expression of a biofilm is also regulated by iron, with a maximum expression occurring at low concentrations (Chaieb et al., 2005).

However, two staphylococcal strains *ica*A+ and *ica*D+ remained biofilm negative even under PIA-expression-stimulating growth conditions. The detection of no biofilm, despite the presence of *ica*, could be due to several reasons such as the inactivation of the *ica* operon by insertion of an IS256 element in the *ica*C gene (Ziebuhr et al., 1999), the action of the i*ca*R repressor (Conlon et al., 2002), or the post-transcriptional regulation (Dobinsky et al., 2003).

Comparison of the CRA test and the results obtained by PCR revealed that among the 17 *ica*A+ and *ica*D+ strains, 16 were slime-producers. In fact, these results

Table 2. The relation between biofilm formation and antimicrobial resistance pattern.

ATB*	Resistance %	Biofilm CRA %		Biofilm TCP %		*ica A%*		*ica D%*	
		Positive (18)	Negative (26)	Positive (15)	Negative (29)	Positive (17)	Negative (27)	Positive (19)	Negative (25)
P	98 %(43/44)	100 % 18 /18)	96% (25 /26)	100% (15 /15)	96.5%(28 /29)	100%(17 /17)	96.2%(26 /27)	100%(19 /19)	96% (24 /25)
OX	79%(35/44)	83% (15 /18)	80.7 (21 /26)	80%(12 /15)	79%(23 /29)	82.3%(14 /17)	77.7%(21 /27)	78.9%(15 /19)	80%(20 /25)
FOX	77%(34/44)	83% (15 /18)	73% (19 /26)	80% (12 /15)	75%(22 /29)	82.3%(14 /17)	74%(20 /27)	78.9% (15 /19)	76%(19 /25)
CN	75%(33/44)	83% (15 /18)	69% (18 /26)	86.6% (13 /15)	68.9%(20 /29)	82.3% (14 /17)	70% (19 /27)	78.9%(15 /19)	72%(18 /25)
TOB	75%(33/44)	83% (15 /18)	69% (18 /26)	86.6%(13 /15)	68.9%(20 /29)	82.3%(14 /17)	70%(19 /27)	78.9%(15 /19)	72%(18 /25)
AK	75%(33/44)	83% (15 /18)	69% (18 /26)	86.6%(13 /15)	68.9%(20 /29)	82.3%(14 /17)	70%(19 /27)	78.9%(15 /19)	72%(18 /25)
VA	0%(0/44)	0% (15 /18)	0% (0 /26)	0% (0 /15)	0%(0 /29)	0%(0 /17)	0%(0 /27)	0%(0 /19)	0%(0 /25)
RA	11%(5/44)	5.5%(1 /18)	15.3% (4 /26)	6.6% (1 /15)	13.7%(4 /29)	5.8%(1 /17)	14.8%(4 /27)	10.5%(2 /19)	12% (3 /25)
FF	7%(3/44)	5.5%(1 /18)	7.6% (2 /26)	6.6%(1 /15)	6.8%(2 /29)	5.8%(1 /17)	7.4% (2 /27)	5.26%(1 /19)	8% (2 /25)
F A	36%(16/44)	16.6% 3 /18)	50%(13 /26)	20%(3 /15)	44.8%(13 /29)	17.6% (3 /17)	48.1%(13 /27)	21%(21 /19)	48% (12 /25)
DA	7%(3/44)	5.5%(1 /18)	7.6(2 /26)	6.6 (1 /15)	6.8%(2 /29)	5.8% (1 /17)	7.4%(2 /27)	5.26%(1 /19)	8% (2 /25)
PT	0%(0/44)	0%(0 /18)	0%(0 /26)	0% (0 /15)	0% (0 /29)	0% (0 /17)	0% (0 /27)	0% (19 /19)	0% (0 /25)
E	73%(32/44)	83% (15 /18)	65.3%(17 /26)	86.6%(13 /15)	65.5% (19 /29)	76.4%(13 /17)	70.3%(19 /27)	73.6%(14 /19)	72%(18 /25)
OFX	73%(32/44)	77.7%(14 /18)	69% (18 /26)	73.3%(11 /15)	72.4%(21 /29)	76.4%(13 /17)	70.3%(19 /27)	73.6%(14 /19)	72%(18 /25)
TE	48%(21/44)	50%(9 /18)	46% (12 /26)	53%(8 /15)	44.8%(13 /29)	52.9%(8 /17)	48.1 % (13% /27)	47.3%(9 /19)	48%(12 /25)
C	7%(3/44)	11.1% 2 /18)	3.8%(1 /26)	6.6%(1 /15)	6.8% (2 /29)	11.7 %(2 /17)	7.3% (1 /27)	10.5%(2 /19)	4%(1 /25)
SXT	34%(15/44)	50% 9 /18)	23%(6 /26)	46.6% (7 /15)	27.5%(8 /29)	52.9 (9 /17)	25.9%(7 /27)	52.6%(10/19)	20%(5/25)

*Antibiotics: Penicilin (P), Oxacillin (OX), Cefoxitin (Fox), Gentamicin (CN), Tobramycin (Tob),Amikacine (AK),Vancomycine (VA),Rifampim(RA), Fosfomycin(FF), FusidicAcid(FA),Clindamycin(DA), Pristinamycin (PT), Erythromycin (E), Ofloxacin(OFX), Tetracycline(TE), Chloramphenicol (C), Triméthoprime/sulfaméthoxazole (SXT).

agree with those obtained by Aricola et al. (2005) and El-Mahallawy et al. (2009) who found a strong correlation bet-ween the *ica*gene positivity and the ability to produce slime by CRA test (P <0.001) compared to the TCP method.

Two isolates of staphylococci were slime+/biofilm- and *ica* A-/*ica*D-. Chokr et al. (2006) reported this phenomenon and suggested that in these strains; variability in the*ica* locus sequence exists, allowing the production of a polysaccharide which reacts with the anti-PIA serum.

Furthermore, *Staphylococcus* spp. isolated from urinary catheters showed high levels of resistance to different classes of antibiotics except vanco-mycine and pristinamycin. They were significantly resistant to penicillin (98%), oxacilline (79%), gentamicine (75%) and ofloxacine (73%). These results are close to those obtained by Touati et al. (2007) who reported that Staphylococci isolated from catheter-related infections are significantly resistant to oxacilline (76.8%), gentamicine (46.4%) and ofloxacine (75%).

Biofilm-forming strains which express *ica* genes are more resistant to antibiotics. This result con-firms that a biofilm adds to the virulence profile of *Staphylococcus* strains isolated from urinary catheters.Biofilms constitute a reservoir of pathogens which are associated with the resistance to antimicrobial agents and cause chronic infections (Seif El-Din et al., 2011; Khan et al., 2011).

The multicellular organization of a bacterium in biofilms gives them the advantage to acquire new genes. The biofilm is a perfect medium for the exchange o fresistance plasmids (Touati et al., 2007) as it combines both the greater probability of contact between cells and the negligible effect of shear forces (Donlan, 2001).

Gilbert et al. (2002) reported that biofilm producers are 10-1000 times less susceptible to antibiotics than are the equivalent cells growing planktonically. A biofilm hampers the penetration of an antimicrobial and the concentrations required to eradicate biofilm-producing bacteria are higher than those required to eradicate strains that did not produce biofilm (Seif El-Din et al., 2011).

In conclusion, our findings show the significant role of *ica* genes as a virulence marker for Staphylococcal isolates. Their association with biofilm-forming strains strongly suggests that expressions of *icaA* and *icaD* genes play a role in the pathogenetic mechanisms o finfections associated with urinary catheters. Hence, in infections caused by biofilm-producing staphylococci, the differentiation with respect to biofilm phenotype might help to modify the antibiotic therapy and preventinfections related to biomedical devices.

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Full Length Research Paper

Hirudo verbana is a source of fungal isolates potentially pathogenic to humans

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As microbial contamination of leeches poses a risk of transmission of pathogens to humans, contact with non-sterile leeches causes a potential hazard to human health. The purpose of this study was to evaluate the mycological purity of the body surface, jaws/pharynx and intestines of the medical leech, *Hirudo verbana*, and the purity of aquarium water within which the leeches were incubated. Leeches were kept without feeding under optimum laboratory conditions recommended for medicinal uses. The strains of fungi were isolated according to our own methods and standard mycological procedures. Of the 150 cultures taken from 50 leeches and 50 samples of water, 152 strains of yeast-like fungi and yeasts belonging to 14 species and 3 genera were identified. The greatest number of fungal species (11) was isolated from the leech jaws, next (10) from the body surface, while the fewest species (8) were found from the samples of water in which the animals were maintained. Fungal isolates belonging to biosafety level two (BSL-2), classified as potential pathogens for humans identified were *Candida albicans*, *Candida ciferrii*, *Candida krusei*, *Candida tropicalis*, *Trichosporon asahii* and *Trichosporon asteroides*. Some other isolates with a decreased pathogenicity potential (BSL-1) also identified were *Candida guilliermondii*, *Candida famata*, *Candida lambica*, *Candida parapsilosis* and *Rhodotorula rubra.* The isolation of a high number of yeast-like fungal strains from *H. verbana* suggests that this medical leech is a vector of potentially pathogenic human fungal species.

Key words: Yeast-like fungi, *Candida* spp., leeches, *Hirudo verbana*, vector.

INTRODUCTION

All leeches are either predatory or parasitic carnivores. The majority of blood-sucking leeches live in fresh water environments. The typical habitat is a eutrophic pond with a muddy substratum with littoral vegetation (Elliott and Kutschera, 2011). Leeches feeding on the blood of numerous aquatic animals such as fishes, amphibians, water birds and mammals (including humans) are a source of pathogens for successive hosts. Additionally various microorganisms living in the natural water reservoir can colonize the leeches (Eroglu et al., 2001; Schulz and Faisal, 2010). More than 650 species of leech have been identified, but only 15 of them are used medically and are classified as medicinal leeches. In Europe, two species of leech, *Hirudo medicinalis* and *H. verbana* are mainly applied in hirudotherapy (Elliott and Kutschera, 2011). During feeding, leeches secrete different biologically and pharmacologically active substances into the wound. Leech saliva contains more than 100 bioactive compounds including coagulation inhibitors, platelet aggregation inhibitors, vasodilators, anti-inflammatory substances and a variety of enzymes, such as collagenase and hyaluronidase (Singh, 2010). Additionally, the application of leeches reduces venous congestion through active absorption of the patient's blood, and passive bleeding after detachment (Porshinsky et al., 2011).

Medical leeches are currently used in various medical specialties, especially in plastic and reconstructive

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surgery, microvascular replantation and traumatology (Porshinsky et al., 2011; Whitaker et al., 2012). The use of H. medicinalis was approved as a medical device by the U.S. Food and Drug Administration (FDA) in 2004. However, the use of leeches can be complicated by infections, especially with the bacterial genus Aeromonas Bacterial infections may vary from minor application in modern medicine greatly increased when wound complications, local abscess and cellulitis, to serious illness such as myocarditis, peritonitis, meningitis, bacteremia and sepsis (Bauters et al., 2007; Yantis et al., 2009; Bourdais et al., 2010). The extensive studies carried out on the gut bacterial flora of medical leeches show that *Aeromonas hydrophila* and *Aeromonas veronii* biovar sobria are the dominant symbiotic species living in the leech digestive tract (Worthen et al., 2006). However, these bacteria are important pathogens to humans. A high incidence of *Aeromonas* infection (2.4 to 36%) has been noted after application of medicinal leeches, despite their external decontamination before their medical use (Bauters et al., 2007). Moreover, infections with other pathogens such as *Serratia marcescens*, *Pseudomonas* spp., *Vibrio fluvialis* associated with medicinal leech therapy have also been reported (Porshinsky et al., 2011). Also, feeding leeches with fresh animal blood during the maintenance and reproductive phases poses the risk of transmission of pathogens to the patients. It has been experimentally demonstrated that many pathogens such as viruses, bacteria, and protozoan parasites from previous blood sources can survive within a leech for many months, and may be transmitted to mammalian hosts (Nehili et al., 1994; Al-Khleif et al., 2011).

A recent study revealed the presence of potentially pathogenic fungal species such as *Candida albicans*, *Candida tropicalis*, *Candida guilliermondii*, *Candida krusei*, on the jaws and body surface of *H. medicinalis* (Biedunkiewicz and Bielecki, 2010). Moreover, some cases of chromoblastomycosis caused by *Fonsecaea* species after wild leech bites have been reported (Ungpakorn and Reangchainam, 2006; Slesak et al., 2011). As a number of fungal species have been reported as etiological agents of human disease, a classification of fungi into biosafety categories was created, and the criteria for attribution to biosafety levels (BSL). In 1996, three BSL categories were formed by the European Confederation of Medical Mycology (de Hoog, 1996). Saprotrophic fungi or plant pathogens able to induce superficial and non-invasive or mild infections belong to BSL-1, whereas BSL-2 contains species that may cause deep, opportunistic mycoses in immunocompromised patients. Pathogens causing superficial infections also classified in BSL-2. Fungi from BSL-3 are pathogens potentially able to cause severe, deep mycoses in otherwise healthy patients. The medical leech used in hirudotherapy is a potential source of many pathogenic microbes for humans. The transmission of pathogens by leeches to patient can occur in several ways. The most common way is inoculation of microorganisms with their saliva into the feeding site. Moreover, blood-sucking leeches require full contact with the wound area, resulting in the contamination of the patient with the microbiota colonizing the body surface and the jaws of the leech. Additionally, the ingested blood in the leech alimentary tract could be re-injected into the host, along with the various microorganisms, by regurgitation during the manipulation of leech removal (Yantis et al., 2009).

The aim of this study was to assess the mycological flora of the body cover, jaws, pharynx and intestine of the widely used leech, *Hirudo verbana*, in hirudotherapy.

MATERIALS AND METHODS

Hirudo verbana

Fifty (50) *H. verbana* leeches with a mean weight of 3.40 g from Natural Medicine Center - Hirudinea (Lodz, Poland) were used. The animals were starved for three months prior to delivery to our laboratory and they were appropriate for use in hirudotherapy. The leeches were placed in five capped glass containers with sterile (boiled) water at a temperature of around 7°C and pH about 8.0. Ten leeches were kept in each container with 5 L of water for three weeks. The water was changed weekly. Water samples were collected after seven days since last change of water.

Isolation of fungal strains from leeches

The fungi were isolated from the water in which the leeches had been kept for 7 days as detailed earlier. Ten 30 ml water samples, without the leeches, were collected from each of the five containers. The samples were concentrated by centrifugation at 5,000 × g for 20 min at 20°C in sterile test tubes and 1 ml volumes of the resulting suspensions were used to inoculate Petri dishes of solid Sabouraud's dextrose medium (SDA) with chloramphenicol. Two Petri dishes were used for each suspension. The plates were incubated at 37°C for 48 to 72 h and then at 24°C for five days. The fungi from the surface of the leech bodies were isolated by washing each leech in 5 ml of Sabouraud dextrose broth (SDB) and incubated at 37°C for 48 to 72 h. This was followed by transfer of 1 ml sample from each of the liquid cultures to the SDA plates and incubated at 37°C for 2 days. The leech jaws, pharynx and intestine were separately prepared under a stereomicroscope, with 20x magnification. All animals were held with sterile gloves and prepared in sterile conditions. Before dissection animals were disinfected externally with alcoholic solution of povidone-iodine accordingly as described by Hokelek et al. (2002). They were then attached to a sterile polystyrene foam plates by sterile pins. The jaws located on the anterior sucker were carefully prepared using a sterile scalpel and tweezers. The preparation of pharynx and intestine was performed according to techniques described by O'Gara et al. (1999) and Worthen et al. (2006), respectively. Three dissected jaws and one pharynx from each leech were collected into one test-tube. The jaws/pharynx and intestine were separately incubated sequentially in 3 ml of SDB and SDA plates at 37°C for 48 to 72 h each. Two SDA palates were used for each transferred 1 ml of SDB.

The mycological examinations were conducted based on procedures introduced in the Department of Diagnostics and Treatment of Parasitic Diseases and Mycoses, Medical University of Lodz (Kurnatowska and Kurnatowski, 2008). The incubated plates were subjected to macroscopic observation. Positive fungal

Table 1. Isolated yeast-like and yeast fungi species from *Hirudo verbana* and water samples.

Species/genus	Place of isolation	BSL* biosafety levels
Candida albicans	IW, JP, BS	2
Candida ciferrii	JP, BS	2
Candida famata	JP, BS	1
Candida guilliermondii	JP, BS	1
Candida krusei	IW	2
Candida lambica	BS	1
Candida parapsilosis	JP, BS	1
Candida tropicalis	IW, BS	2
Lipomyces starkeyi	IW, JP	ND
Rhodosporandium sp.	JP, BS	ND
Rhodotorula rubra	IW, BS	1
Schizosacharomyces sp.	JP, BS	ND
Trichosporon asahii	IW	2
Trichosporon asteroides	IW	2
Trichosporonoides oedocephalis	JP	ND
Trichosporonoides sp.	JP	ND
Yarrowia lipolytica	IW, JP	ND

IW - incubation water, BS - body surface, JP - jaws/pharynx, ND - BSL not defined, *according to de Hoog (1996).

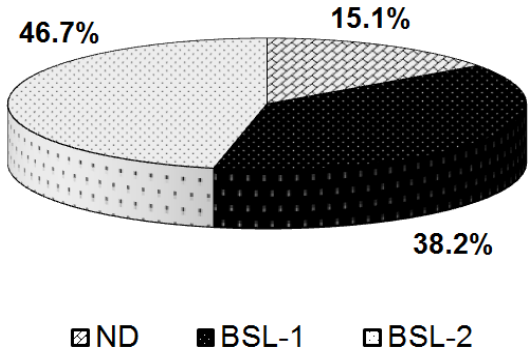

Figure 1. The percentages of fungal strains belonging to different biosafety levels (BSL) isolated from all cultures.

macrocultures were subcultured on SDA for the isolation of a pure, single colony for species identification. In order to evaluate the morphological and biochemical characteristics of the isolated fungi, the following methods were applied: direct microscopic slides, microculture technique, selective media such as Nickerson's medium and biochemical tests for example, auxanograms. Initially, the isolated fungi were determined to species/genus level on the basis of keys by De Hoog et al. (2000) and Kurtzman and Fell (2000). The axenic strains were definitively identified on the basis of the biochemical characteristics of their carbohydrate assimilation using the API 20C AUX test, according to the principle of numerical identification (Analytical Profile Index, BioMeriéux, Lyon 1990).

Statistical analysis

The differences between groups were compared by the Chi-square test or Fisher's exact test. Values of $p < 0.05$ were taken as significant. All calculations were performed using STATISTICA v. 10.0 software.

RESULTS

Fungi were found in all samples from the body surface, the leech jaws/pharynx and water samples. No fungi were recorded in the leech intestines. The presence of yeasts and yeast-like fungal species isolated from examined materials is shown in Table 1. Mycological examinations of 150 cultures from 50 leeches and 50 samples of water identified 152 fungal strains of yeast-like fungi, and yeasts belonging to 14 species and 3 genera (Table 1). A similar number of fungal species was found in the three materials. Eleven (11) species were isolated from the leech jaws/pharynx, 10 from the body surface, and 8 species were found from the samples of water in which the animals were maintained.

As seen in Table 1, fungi classified as potential human pathogens (BSL-2), species with a lowered potential of pathogenicity (BSL-1) and saprotrophic fungi (ND) were detected. Saprotrophic fungi not pathogenic to humans and animals are not classified to any BSL categories. Five species belonging to BSL-2 were isolated from the water samples. Three species with BSL level 2 were found in macrocultures from the body surface of the leeches and two from their jaws/pharynx. No statistically significant differences were found in the number of species assigned to the BSL-2 category isolated from the aforementioned materials ($P>0.05$). Nearly half of the 152 detected isolates (46.7%) belonged to BSL-2 (Figure 1). Isolates from BSL-2 and BSL-1 were found to be, respectively, 3 and 2.5 times more common than

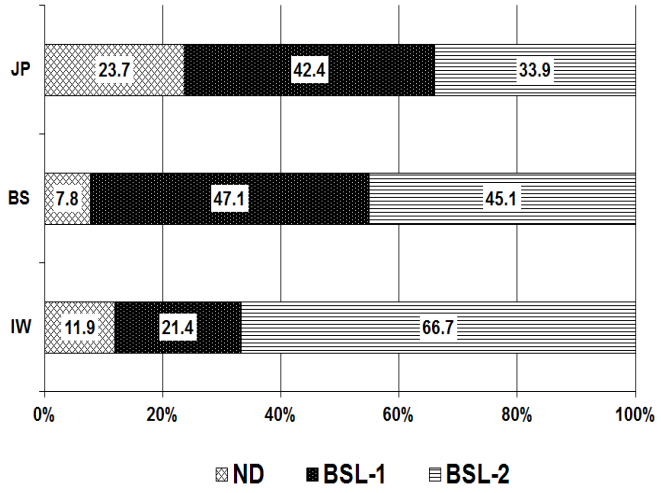

Figure 2. The percentages of fungal strains from different BSL categories isolated from water (IW), jaws/pharynx (JP) and body surface (BS) of *Hirudo verbana.*

saprotrophic strains (ND). Figure 2 shows the percentage distribution of fungal strains according to BSL categories and their place of isolation. There was a significant difference in number fungal strains isolated from the different biological samples (Chi^2 = 15.058, df = 4, P = 0.00458).

In the water samples, the largest number of strains (28 strains; 18.4% of total isolates) belonging to BSL-2 was detected, and it was significantly different from the numbers of strains from BSL-2 isolated from the jaws/pharynx (Chi^2 = 10.564, df = 1, P = 0.00115) and body surface of leeches (Chi^2 = 4.326, df = 1, P = 0.0375). Similar numbers of BSL-1 isolates (P>0.05) were identified from cultures of the jaws/pharynx (25) and body surface (24), while only 9 strains from BSL-1 class were discovered in water samples. The most frequently detected fungi were *C. albicans* (20.4%), *C. parapsilosis* (17.1%) and *C. tropicalis* (13.2%). *C. albicans* strains were found in three examined materials; 31 strains were isolated from the body surface of the leech (11), its jaws/pharynx (13) and also the water samples (7).

Six biochemical phenotypes of *C. albicans* were assigned with different numerical assimilation profiles (Analytical Profile Index, bioMérieux, Lyon 1990), and among the isolates, two codes dominated: 2576154 (35.5%) and 2576174 (29.0%) as shown in Table 2. From five biotypes of *C. tropicalis*, phenotype 2556375 dominated, whereas code 6756175 was isolated most often from strains of *C. parapsilosis*. Some species occurred less frequently: *C. guilliermondii*, *C. famata*, *C. ciferri*, *Rhodotorula rubra*, *Lipomyces starkeyi*, *Yarrowia lipolytica* and *Trichosporonoides oedocephalis*. Other species/genera such as *C. krusei*, *C. lambica*, *Trichosporon asahii* and *Trichosporon asteroides*, *Rhodosporandium* sp., and *Schizosacharomyces* sp. were isolated occasionally.

DISCUSSION

In this study, the six fungal species isolated from water samples, body surface and jaws/pharynx of *H. verbana* were classified as BSL-2 and five isolates as BSL-1, despite the leeches being kept in sterile laboratory conditions. A study by Biedunkiewicz and Bielecki (2010) also identified potentially pathogenic *Candida* species on the body surface and jaws of *H. medicinalis*. It should be noted that the leeches used in this and the study aforementioned were cultured in two different leech farms, and so had never been in contact with the natural environment. Fungal colonization of these leeches was probably a consequence of the non-sterile conditions associated with breeding, growing or transport. This could be because *Candida* species are ubiquitous that is, may be found in fresh water, soil, fruit, animals or humans (Schauer and Hanschke, 1999) and are the most common fungal pathogens that infect humans.

The sources of microbial contamination leeches at the leech farms could be leech tanks and water, the ground where leech cocoons are incubated, blood meal given to growing animals or farm workers, especially their hands. *C. albicans* is considered an opportunistic pathogen which frequently colonises human skin (Kim and Sudbery, 2011). It was confirmed that human hands are an important route for transmission of fungi from one person to another, and from people to inanimate surfaces, and hand hygiene still remains the major preventive measure against nosocomial infections (Yildirim et al., 2007).

C. albicans strains were detected from all examined materials with the exception of *H. verbana* intestine. Because most of these strains were isolated from leeches kept in laboratory conditions, it can be assumed that the animals were first colonized by them during farming or transport and then the sterile water used has been secondarily contaminated. The absence of fungal strains in leech intestines is most likely due to the intensive colonization of their digestive tract by symbiotic bacteria, which inhibit the growth of other microorganisms (Worthen et al., 2006). In our study, numerous bacteria belonging to the genus *Aeromonas* were isolated from intestinal cultures. Among six of the *C. albicans* strains isolated from *H. verbana*, two biochemical phenotypes (2576154, 2576174 - API-20C AUX) were predominant and they were detected both from cultures of jaws/pharynx and body surface, as well as from water samples. These same *Candida* strains, with numerical assimilation profiles 2576174 and 2576154, were found most frequently by other authors in both people with fungal skin colonisation and candidosis (Williams et al., 2000; Glowacka, 2002). In particular, a strain of *C. albicans* coded 2576174 is the most common strain observed in people with symptomatic candidoses (Williams et al., 2000). The API-20C AUX test is used successfully in epidemiological studies (Kurnatowska and Kurnatowski, 2008). A comparative analysis of the assimilation phenotypes of strains of the same species

Table 2. Numerical identification codes (Analytical Profile Index, bioMérieux, Lyon, 1990) of isolated strains (n = 129) belonging to BSL class 1 or 2 from biological materials of *Hirudo verbana* and water samples.

Species (No. of strains)	Code	Number of strains	
		n	% ± SD
Candida albicans (n = 31)	2576154	11	35.5 ± 8.59
	2576174	9	29.0 ± 8.15
	2576074	5	16.1 ± 6.60
	2566174	3	9.7 ± 5.31
	2572174	2	6.5 ± 4.42
	2576174	1	3.2 ± 3.16
Candida ciferrii (n = 11)	6701366	6	54.5 ± 15.01
	6671366	4	36.4 ± 14.07
	6643176	1	9.1 ± 8.67
Candida famata (n = 9)	2576773	5	55.6 ± 16.56
	6756373	2	22.2 ± 13.85
	6756773	2	22.2 ± 13.85
Candida guilliermondii (n = 9)	6756377	6	66.7 ± 15.71
	6676371	3	33.3 ± 15.71
Candida krusei (n = 3)	1000005	3	100 ± 0.0
Candida lambica (n = 2)	2400004	2	100 ± 0.0
Candida parapsilosis (n = 26)	6756175	9	34.6 ± 9.32
	6756135	7	26.9 ± 8.60
	2756175	5	19.2 ± 7.72
	2656175	3	11.5 ± 6.26
	6756171	1	3.8 ± 3.75
	6756131	1	3.8 ± 3.75
Candida tropicalis (n = 20)	2556375	7	35.0 ± 10.66
	2556175	5	25.0 ± 9.68
	2576175	5	25.0 ± 9.68
	6556175	2	10.0 ± 6.71
	2552174	1	5.0 ± 4.87
Rhodotorula rubra (n = 12)	6402073	7	58.3 ± 14.23
	2610062	5	41.7 ± 14.23
Trichosporon asahii (n = 5)	2744775	3	60 ± 21.91
	2767735	2	40 ± 21.91
Trichosporon asteroids (n = 1)	3364325	1	100 ± 0.0

isolated from different parts of human body or several persons can help determine intra- and inter-human transmission of pathogenic fungi. Moreover, horizontal transmission (environment-people) of *C*. *albicans* strains was confirmed on the basis of the identity of digital codes of strains isolated from human skin lesions and the sanitary devices with which they had contact (Glowacka, 2002). In our studies, *Candida* strains (*C. albicans* and *C. tropicalis*) detected in water samples were found to have the same numerical assimilation profiles as those seen in cultures of biological materials of *H*. *verbana.* Hence, the water in which the leeches were being kept was contaminated by strains colonizing the body surface and/or jaws. Among the non- *C. albicans* leech isolates,

predominant assimilation phenotypes of *C. tropicalis* and *C. parapsilosis* were frequently detected in human biological materials collected from patients with nosocomial mycoses (Ng et al., 2001). Our results suggest that fungal assimilation biotypes colonising leech jaws/pharynx and body surfaces may be the cause of wound complications occurring during hirudotherapy. Hence, the maintenance of sterile conditions for the culture, transport and storage of medical leeches is of paramount importance.

Today, leech therapy is indicated in plastic and reconstructive surgery to relieve venous congestion and to improve the microrevascularization of flaps or replants, with a 60 to 83% increase in success rate (Bourdais et al., 2010; Whitaker et al., 2012). Moreover, the postoperative application of leeches carries the risk of microbial infection. In the presence of infection as a complication of the medicinal use of leeches, the success rate for flap salvage may decrease to over 30% (Whitaker et al., 2012). Hence, the sterility of the leeches used in hirodotherapy is a fundamental aspect of patient safety. The results of the present study underline the importance of maintaining sterile conditions not only during storage of medical leeches but also during their development and growth in leech farms.

Conclusion

The identification of fungi and yeast-like fungi on the body surfaces and jaws/pharynx of *H. verbana* kept under optimum laboratory conditions implies that this leech can act as a vector of these potential human pathogens.

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Full Length Research Paper

Antifungal activity of secondary metabolites of *Pseudomonas fluorescens* isolates as a biocontrol agent of chocolate spot disease (*Botrytis fabae*) of faba bean in Ethiopia

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Pseudomonas fluorescens **isolates possess a variety of promising properties of antifungal activity of secondary metabolites which make it as a biocontrol agent. In the present study, 12 isolates of *P. fluorescens* were isolated from rhizospheric soil of faba bean crop evaluated for their antagonistic activity against chocolate spot disease (*Botrytis fabae*) of faba bean. *P. fluorescens* 10 (Pf 10) (88.1%) showed high antagonistic activity against *B. fabae*. All isolate of *P. fluorescens* were successfully employed in controlling chocolate spot disease of faba bean due to their antifungal metabolites. The antifungal compounds were extracted from all *P. fluorescens* isolates with equal volume of ethyl acetate, hexane and methanol. The antifungal compounds extracted with ethyl acetate, hexane and methanol from *P. fluorescens* 3 (Pf 3), *P. fluorescens* 8 (Pf 8), and *P. fluorescens* 3 (Pf 3), isolates at 0.1% concentration completely inhibited the mycelial growth of the pathogen respectively. Bio-primed faba bean seeds with isolates of *P. fluorescens* 9 (Pf 9) and *P. fluorescens* 10 (Pf 10) evaluated against *B. fabae in vivo* (pot culture) indicated the inhibitory effects to the pathogen and also showed the inducing properties to enhance the immune system of crop. Therefore, it can be concluded that the use of *P. fluorescens* 9 (Pf 9) and *P. fluorescens* 10 (Pf 10) of isolates could inhibit the mycelial growth and reduced the disease incidence, severity and infection processes of *B. fabae* and simultaneously increase the plant growth performance and yield of faba bean. These isolates can be used as potential biocontrol agents against *B. fabae* and also used as biofertilizers for the production of faba bean.**

Key words: Antifungal, Biocontrol, *Botrytis fabae*, Faba bean, *Pseudomonas fluorescens*.

INTRODUCTION

There is an urgent need to improve *Vicia faba* yield, since this plant remains an important part of the diet of both humans and domestic animals in many parts of the world, because of its high nutritive value in both energy

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and protein contents. Furthermore, faba bean supplies an important benefit to the crop by fixing atmospheric nitrogen in symbiosis with *Rhizobium leguminosarum* thus, reducing costs and minimizing impact on the environmental, which is why increasing the plant production is one of the major targets of the agricultural policy in several countries (Mahmoud et al., 2004).

However, this crop is subjected to many abiotic and biotic stresses that seriously compromise the final yields. Among the menacing biotic stresses, chocolate spot, caused by *Botrytis fabae*, is a worldwide disease capable of devastating the unprotected faba bean, result in harmful effects on growth, physiological activities and yield. Chocolate spot disease of faba bean is the most wide spread and destructive disease in Ethiopia with yield reductions of up to 61% on susceptible cultivars (Dereje and Beniwal, 1987). The problem of adequately protecting plants against the fungus by using fungicides has been complicated by development of fungicidal resistance and many chemicals traditionally used to control chocolate spot disease is less effective (Harrison, 1984), giving only partial disease control, high cost of their use and /or adverse effects on growth and productivity of faba bean as well as on the accompanying microflora (Khaled et al., 1995). Therefore, controlling *B. fabae* by biocontrol agents seemed to be better and preferred than the chemical control (Mahmoud et al., 2004). Bio-priming, a seed treatment system that integrates the biological and physiological aspects of disease control, involves coating the seed with fungal or bacterial biocontrol agents (El-Mougy and Abdel-Kader, 2008).

The diversity and beneficial activity of the plant-bacterial association and its understanding is important to sustain agro-ecosystems for sustainable crop production (Germida et al., 1998). *Pseudomonas fluorescens* is a gram-negative, rod-shaped, and non-pathogenic bacterium that is known to inhabit primarily the soil, plants, and water (Peix et al., 2009). It derives its name from its ability to produce fluorescent pigments under iron-limiting conditions (Baysse et al., 2003). These bacteria belong to soil microorganisms that develop one of the very important soil processes of denitrification. Biological control is a promising approach for management of plant diseases. Biocontrol agents of *P. fluorescens* are well characterized for their ability to produce antimicrobial compounds (Haas and De´fago, 2005).

The concept of biocontrol of plant diseases includes disease reduction or decrease in inoculum potential of a pathogen brought about directly or indirectly by other biological agencies (Johnson and Carl, 1972). Outside the host, the biocontrol agent may be antagonistic and thereby reduce the activity, efficiency and inoculum density of the pathogen through antibiosis, competition and predation/hyper parasitism. This leads to a reduction in inoculum potential of the pathogens (Baker, 1977). Bio-priming, a seed treatment system that integrates the biological and physiological aspects of disease control, involves coating the seed with fungal or bacterial biocontrol agents (El-Mougy and Abdel-Kader, 2008).

The addition of Carboxymethyl cellulose (CMC) or pectin to bio-primed seeds enhanced the antagonists' ability to grow and survive competitively. In addition, they had no effect on seed germination (Elzein et al., 2006). The present study was designed to isolate certain rhizospheric bacteria of *P. fluorescens* for their antagonistic and antifungal activity of secondary metabolites against chocolate spot disease (*B. fabae*) to reduce the disease incidence and severity in order to increase yield of faba bean.

MATERIALS AND METHODS

Soil sample collection and bacterial isolation

Rhizospheric soil samples were collected from fields growing faba bean (*Vicia faba* L.) from five localities area of Selale zones, Oromia Region, Ethiopia. The soil samples were brought to Mycology Laboratory, Addis Ababa University. 10 g of rhizosphere soil sample was suspended in 90 ml of sterile distilled water. Samples were serially diluted up to 10^5 to 10^6 and 0.1 ml of sample was spread on King's B medium plates (King et al., 1954). After incubation at 28°C for 48 h, the plates were exposed to UV light at 365 nm for few seconds and the colonies exhibiting the fluorescence were picked up and purified on King's B medium plates and 12 *P. fluorescens* isolates (Pf 1) were isolated and they were designed as Pf 1 up to Pf 12 for further studies.

Source of faba bean and chocolate spot disease

Faba bean seed used in the present work was obtained from Holleta Agriculture Research Centre, Ethiopia. Three varieties of faba bean seed were provided (such as: NC 58 susceptible variety, Moti moderate variety and ILB 938 relative resistant variety). One isolate of *Botrytis fabae* was obtained from Holleta Agricultural Research Centre, Ethiopia. This strain was isolated from the leaf of infected faba bean crops grown from Holleta areas.

In vitro evaluation of bacterial antagonit against the test pathogen

All *P. fluorescens* isolates were assessed for potential antagonistic activity against *B. fabae* on King's B agar using dual culture technique (Rangeshwaran and Prasad, 2000). An agar disc (4 mm) was cut from an actively growing (96 h) *B. fabae* culture and placed on the surface of fresh King's B agar medium at the center of the Petri plates. A loopful of actively growing *P. fluorescens* isolates was placed opposite to the fungal disc and the *P. fluorescens* isolates on the plate were streaked at four locations, approximately 3 cm from the center. Plates inoculated with pathogen and without bacteria were used as control. All *in vitro* tests of antagonism were performed triplicates, with new co-inoculations used each time. Plates were incubated at room temperature for 7 days. Degree of antagonism was determined by measuring the radial growth of pathogen with bacterial culture and control. The percentage of mycelial growth inhibition was calculated by the following equation (Riungu et al., 2008):

$$\text{Percent of Inhibition} = \frac{C - T)}{C} X\ 100$$

Where, C= Radial growth of fungus in control plates (mm) and T= radial growth of fungus on the plate inoculated with antagonist (mm).

Extraction of secondary metabolites of *P. fluorescens* isolates

All *P. fluorescens* isolates were grown in 100 ml of King's B media in 250 ml conical flask in orbital shaker at 28°C and 120 rpm, for 96 h. The culture was centrifuged at 10,000 rpm for 15 min to get the cell-free filtrate (Tripathi and Johri, 2002). Secondary metabolites were extracted by partitioning with organic solvents such as: ethyl acetate, hexane and methanol the three solvents (Tripathi and Johri, 2002). The antifungal compounds were extracted from cell-free broth with equal volume of ethyl acetate, hexane and methanol (1:1:1) and the extract was separated from the aqueous by using separating funnel and then evaporated in a rotary evaporator at 45, 60 and 65°C at 121 rpm to ensure complete solvent removal respectively. The extracted secondary metabolites without concentration were tested for their efficacy against pathogens by poison food technique (Nene and Thapliyal, 1973). The concentrations of extracted secondary metabolite (0.1%) (25 µm) were prepared and poured on King's B agar medium with mixed, before a 4 mm disc of *B. fabae* culture was inoculated at the center of each plate; three replications were maintained for each treatment and the Petri dishes were incubated at 28°C. King's B medium plates with only solvent served as control. After full growth of the control plate's size of colony, diameter measured in mm and percentage inhibition of mycelial growth was calculated using the formula (Mohana and Raveesha, 2007):

$$\text{Percent of Inhibition} = \frac{(C - T)}{C} X\ 100$$

Where, C = Average increase in mycelial growth in control plate and T=Average increase in mycelial growth in treatment plate.

Greenhouse experiment

Preparation of fungal inoculum

The inoculums of *B. fabae* were prepared from old culture grown on faba bean seed dextrose agar at 28°C. Conidia were harvested by scraping, transferred to sterilized distilled water and filtered through nylon mesh. Spore suspensions of *B. fabae* were adjusted to 2.5 × 10^5 spores mL^{-1} with sterile distilled water using a haemocytometer as described by Derckel et al. (1999).

Preparation of bacteria inoculum

P. fluorescens isolates were grown for 48 h in King's B (KB) broth medium, and then cells were harvested by centrifugation. Bacterial cell were resuspended in sterile distilled water and the concentration adjusted to 10^9-10^{10} cells/ml (El-Mougy and Abdel-Kader, 2008).

Bio-priming of faba bean seeds

Carboxymethyl cellulose (CMC) and pectin were used as adhesive polymers for the bio-priming process of three varieties of faba bean seeds with antagonistic biological agents. Two isolates of *P. fluorescens* were resuspended in sterile distilled water and the concentration adjusted to give 10^9-10^{10} cells/ml. 10 g of either CMC or pectin was resuspended in 1 L of *P. fluorescens* isolates suspensions. Seeds of faba bean (at the ratio of 500 g/L) were imbibed in each of the prepared priming solutions for 16 h (Jensen et al., 2004). The bio-primed seeds were then air-dried on filter paper for 1 h and stored in a refrigerator at 5°C until required. Another group of surface-sterilized faba bean seeds (70% ethanol for 2 min) was prepared as control treatments (El-Mougy and Abdel-Kader, 2008).

Pot experiments

The experiment were designed under greenhouse conditions in Ecology and Ecophysiology Greenhouse, Addis Ababa University in March 2012, using pots (21 cm) containing 4 kg of sterilized loamy clay soil. First, soils were infested with 20 ml of *B. fabae* spore suspension (2.5 × 10^5 spores/ml) by soil drenching (Haggag et al., 2006). The pots were irrigated for 7 days before bio-control agent inoculation. Afterward, four of the bio-primed faba bean seeds were sown in each pot. The experiment included the following treatments: 1) non-infested soil (control); 2) soil only treated with *B. fabae*; 3) *B. fabae* + *P. fluorescens* isolates (P f 9 and P f 10), separately. Pots were kept under greenhouse conditions until the end of the experiment (Abd-El-Khair et al., 2010).

Disease assessment

The disease incidence (DI) and disease severity (DS) of chocolate spot disease were recorded at the 50 and 70th day after planting of faba bean in *in vivo* condition in green house. The disease severity of chocolate spot disease was recorded at 50 and 70 days from sowing under natural infection by using the scale of Bernier et al. (1993) as follows:

1 = No disease symptoms or very small specks (highly resistance); 3 = few small discrete lesions (resistant); 5 = some coalesced lesion with some defoliation (moderate resistant); 7 = large coalesced sporulating lesions, 50% defoliation and some dead plant (Susceptible); 9= Extensive lesions on leaves, stems and pods, severe defoliation, heavy sporulation, stem girdling, blackening and death of more than 80% of plants (Highly susceptible).

Chocolate spot disease severity was assessed according to the scale of Bernier et al. (1984).

$$\text{Disease severity \%} = \frac{(nxv)}{9n} x100$$

Where, (n)= Number of plants in each category; (v)= Numerical values of symptoms category; (N)= Total number of plants; (9)= Maximum numerical value of symptom category.

The disease incidence of chocolate spot as a disease percentage was determined after 50 and 70 days from sowing the first treatment according to the following formula:

$$\text{Disease incidence} = \frac{\text{Number of infected leaf lets}}{\text{Total number of tested leaflets}} x100$$

The efficacy percentage (E %) of *P. fluorescens* (P f9 and P f10) in reducing disease severity percentage of faba bean was assessed according to the equation adapted by Rewal and Jhooty (1985) as follow:

$$\text{Percent of Inhibition} = \frac{\text{\% disease severity in control} - \text{\% disease severity treatment}}{\text{\% Disease severity in control}}$$

Figure 1. *Pseudomonas fluorescens* was isolated based on their pigment production under UV light at 365 nm.

Figure 2. *Pseudomonas fluorescens* isolates was confirmed again under UV light at 365 nm.

Pathogenicity test

Re- isolation of the pathogen

B. fabae was re-isolated from the leaf lesion of the control plants in the *in vivo* experiment. Leaf lesions were cut into pieces and surface sterilized with 70% ethanol for 2 min and rinsed three with sterile water in Petri plates. Pieces were dried with sterile filter paper, plated on faba bean seed extract dextrose agar (FDA) medium and incubated at 28°C for 7 days. The fungus was subculture for purification, and identification was done using microscopes observation of the spore morphology and comparison with the original culture.

Data analysis

All the measurements were replicated three times for each assay and the results are presented as mean ± SD and mean ± SE. IBM SPSS 20 version statistical software package was used for statistical analysis of percentage inhibition and disease incidence and disease severity in each case.

RESULTS

Isolation of *P. fluorescens*

During this research work, 12 *P. fluorescens* were isolated from rhizospheric soil of healthy faba bean from five locality of Oromia region, Ethiopia, on King's B medium and observed under UV light at 365 nm for few seconds as shown in Figure 1. Then, it was purified again on same medium and observed under UV light as indicated in Figure 2. All the rhizospheric isolates were named as Pf 1 to Pf 12 as indicated in Table 1 (*P. fluorescens* isolate 1 = P f1, *P. fluorescens* isolate 2 = P f2, *P. fluorescens* isolate 3 = P f3, *P. fluorescens* isolate 4= P f4, *P. fluorescens* isolate 5 = P f5, *P. fluorescens* isolate 6 = P f6, *P. fluorescens* isolate 7 = P f7, *P. fluorescens* isolate 8 = P f8, *P. fluorescens* isolate 9 = P f9, *P. fluorescens* isolate 10 = P f10, *P. fluorescens* isolate 11 = P f11, *P. fluorescens* isolate 12 = P f12) and maintained on Nutrient Agar slants for further testing and biochemical production test. Spore morphology of *B. fabae* and the sporulation spores attachment to mycelia were observed under microscope by execution of slide culture as indicated in Figure 3.

In vitro evaluation of bacterial antagonistic activity against the test pathogen

The results of *in vitro* evaluation and testing of *P. fluorescens* isolates showering antagonistic activities towards *B. fabae* are shown in Table 1 and Figure 4. Inhibition was clearly discerned by very limited growth of fungal mycelium in the inhibition zone surrounding a bacterial colony. The antagonistic effects of *P. fluorescens* isolates against *B. fabae* were in the range of 84.1- 88.1%. Pf 10 gave the maximum inhibition about 88.1 %, followed by Pf 9 (88.0 %). Control plates were not treated with isolates of *P. fluorescens* completely covered by the *B. fabae*.

Antifungal activity of ethyl acetate extracts of secondary metabolites of *P. fluorescens* isolates against *B. fabae*

The results for all the *P. fluorescens* isolate are shown in Table 2. Ethyl acetate extracts of the isolate Pf 3 completely inhibited the growth of *B. fabae.* The maximum inhibition of mycelia growth of *B. fabae* was observed in extracts of Pf 9 (86.30%) and Pf 10 (85.20).

Antifungal activity of hexane extracts of secondary metabolites of *P. fluorescens* isolates against *B. fabae*

The result shown in Table 3 indicates that Pf 8 at 0.1% concentration totally inhibited the growth of mycelia and

Table 1. Effect of antagonistic activity of *P. fluorescens* isolates treatments against the leaner mycelial growth of *Botrytis fabae in vitro* tests.

***P. fluorescens* isolate**	**Antagonistic effect against *Botrytis fabae***	
	Mycelial diameter (cm) (Mean ± SD)	**Inhibition (%)**
Pf 1	2.30 ±0.26	87
Pf 2	2.63 ±0.32	85.4
Pf 3	2.43 ±0.21	86.5
Pf 4	2.86 ±0.12	84.1
Pf 5	2.73 ±0.31	84.8
Pf 6	2.63 ±0.49	85.4
Pf 7	2.23 ±0.25	87.6
Pf 8	2.40 ±0.36	86.7
Pf 9	2.20 ±0.00	88
Pf 10	2.13 ±0.15	88.1
Pf 11	2.46 ±0.50	86.3
Pf 12	2.80 ±0.20	84.8
Control	9.00 ±0.00	-

SD= standard deviation.

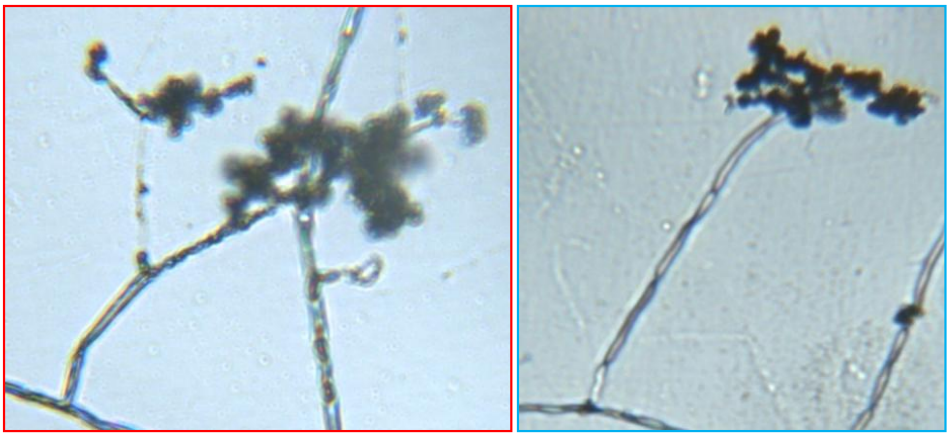

Figure 3. Conidiophore of *Botrytis fabae* (Bran|CHED dichotomously).

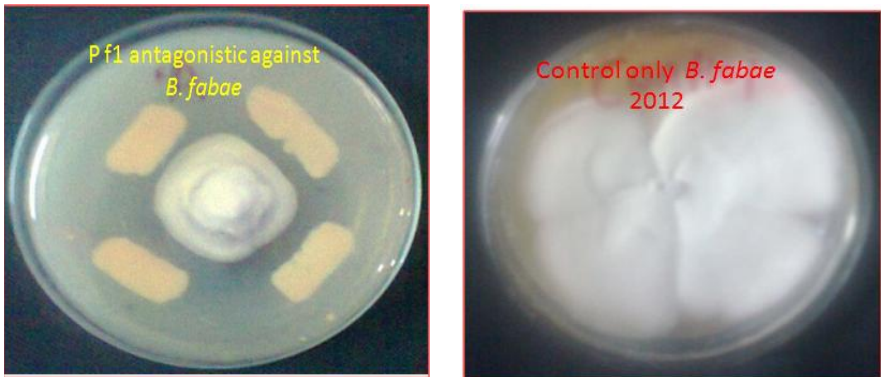

Figure 4. Dual culture of *Pseudomonas fluorescens* isolates with *B. fabae* on King's B medium.

Table 2. Percentage of inhibition of ethyl acetate extract of *Pseudomonas fluorescens* isolates metabolites at 0.1 % concentration against *B. fabae.*

Antifungal compounds of *P. fluorescens*	*B. fabae* isolates	
	MG (mm) (Mean ± SE)	INH %
Pf 1	15.33 ±1.45	82.96
Pf 2	18.00 ±2.08	80.00
Pf 3	No growth	100
Pf 4	14.67 ±1.45	83.70
Pf 5	16.33 ±0.88	81.85
Pf 6	14.67 ±1.76	83.70
Pf 7	15.33 ±1.45	82.96
Pf 8	14.83 ±0.60	83.52
Pf 9	12.33 ±0.88	86.30
Pf 10	13.33 ±0.88	85.20
Pf 11	15.67 ±1.20	82.60
Pf 12	16.67 ±1.20	81.48
Control	90.00± 0.00	-

MG= Mycelial growth; INH= inhibition over control; SE=Standard error of mean.

Table 3. Percent of inhibition of hexane extract of secondary metabolites of *Pseudomonas fluorescens* isolates at 0.1 % concentration against *B. fabae.*

Antifungal compounds of *P. fluorescens*	*B. fabae* isolates	
	MG (mm) (Mean ± SE)	INH %
Pf 1	18.67 ±2.33	79.26
Pf 2	17.00 ±1.73	81.11
Pf 3	18.67 ±1.20	79.26
Pf 4	20.67 ±1.45	77.04
Pf 5	22.67 ±0.88	74.81
Pf 6	21.33 ±1.86	76.30
Pf 7	23.00 ±1.53	74.44
Pf 8	No growth	100
Pf 9	13.00 ±0.58	85.60
Pf 10	16.00 ±2.52	82.22
Pf 11	20.33 ±1.20	77.41
Pf 12	18.00 ±1.53	80.00
Control	90.00 ±0.00	-

MG= Mycelial growth; INH= inhibition over control; SE=Standard error of mean.

the highest percent of inhibition on the growth of *B. fabae* was obtained with extracts of Pf 9 (85.60%) followed by P f10.

Antifungal activity of methanol crude extracts of secondary metabolites of *P. fluorescens* isolates against *B. fabae*

The effect of extracellular metabolites extracts of *P. fluorescens* isolates on the growth of *B. fabae* is shown in Table 4. The two effective extracts of Pf 3 and Pf 10 showed complete inhibition and highest percentage of inhibition of the mycelial growth of *B. fabae* respectively.

Pot experiments

Evaluation of bio-primed seeds of faba bean treatments with *P. fluorescens* isolates were the suppression of *B. fabae* disease incidence and severity investigated under artificial inoculation conditions (Table 5). The result of disease incidence, severity treatment and efficacy are shown in Table 5. Disease symptoms attributed to *B. fabae* were

Table 4. Percentage of inhibition of methanol extract of secondary metabolites of *Pseudomonas fluorescens* isolates at 0.1 % concentration against *B. fabae.*

Antifungal compounds of *P. fluorescens*	*B. fabae* isolates	
	MG (mm) (Mean ± SE)	INH %
Pf 1	19.33 ±1.20	78.52
Pf 2	20.00 ±1.53	77.78
Pf 3	No growth	100
Pf 4	18.00 ±0.58	80.00
Pf 5	17.33 ±1.67	80.74
Pf 6	22.00 ±.58	75.56
Pf 7	20.00 ±1.73	77.78
Pf 8	22.33 ±0.88	75.19
Pf 9	14.00 ±2.08	84.44
Pf 10	13.67 ±2.52	84.82
Pf 11	25.00 ±1.53	72.22
Pf 12	16.00 ±1.53	82.22
Control	90.00 ±0.00	-

MG= Mycelial growth; INH= inhibition over control; SE=Standard error of mean.

Table 5. Disease severity and incidence of chocolate spot disease (*Botrytis fabae*) on faba bean leaves treated with *P. fluorescens* isolate 9 and *P. fluorescens* isolate 10 under greenhouse condition.

Treatments and Controls	After 50 days			After 70 days		
	DS (%)	Efficacy(%)	DI (%)	DS (%)	Efficacy(%)	DI (%)
Pf 9 NC 58	11.11	40.01	25.00	11.11	57.14	41.67
Pf 9 Moti	3.70	66.70	8.33	11.11	40.01	33.33
Pf 9 ILB 938	3.70	66.70	8.33	3.70	80.02	16.67
Pf 10 NC 58	3.70	80.02	16.67	3.70	85.73	33.33
Pf10 Moti	3.70	66.70	8.33	3.70	80.02	16.67
Pf10 ILB 938	3.70	66.70	8.33	3.70	80.02	16.67
Negative Control NC 58	18.52	-	66.67	25.93	-	75.00
Negative Control Moti	11.11	-	33.33	18.52	-	66.67
Negative Control ILB 938	11.11	-	16.67	18.52	-	58.33
Positive Control NC 58	3.70	-	-	3.70	-	-
Positive Control Moti	3.70	-	-	3.70	-	-
Positive Control ILB 938	3.70	-	-	3.70	-	-

DS = disease severity, DI= disease incidence.

observed slightly on faba bean plants grown in soil artificially infested with bio-primed seeds of faba bean with two *P. fluorescens* isolates (Pf 9 and Pf 10) in pot experiment as compared with the control. Bio-primed seeds of faba bean Moti and ILB 938 with Pf 9 and Pf 10 showed lowest disease severity compared with the untreated plants after 50 days. In general, two isolates of *P. fluorescens* effectively reduced the disease on the susceptible (NC 58), moderately resistant (Moti) and relative resistant (ILB 938) whereas, disease incidence of bio-primed seeds of faba bean Moti and ILB 938 with P f9 and P f10 had the lowest compared with the untreated ones after 50 day. Disease severity was constantly delayed on NC 58, Moti and ILB 938 varieties during the observation period after 70 days. Disease incidence after 70 days, were lowest on 16.67% ILB 938 varieties with Pf 9 and Pf 10 compared with the untreated.

DISCUSSION

In the present study, *in vitro* evaluation of all *P. fluorescens* isolates treatments reduced the mycelial growth of *B. fabae* on King's B medium. It has been observed that the mycelial growth was reduced due to the production of secondary metabolites which inhibited growth of *B. fabae*. Similarly, *P. fluorescens* was shown

to effectively inhibit *R. solani* and *P. oryzae* by agar plate method (Rosales et al., 1995).

The present results of all *P. fluorescens* isolates showed the maximum inhibition (88.1%) of mycelial growth of *B. fabae. P. fluorescens* isolated from rhizosphere of organic farming area is effective against *Rhizoctonia solani* (Anitha and Das, 2011). *P. fluorescens* strain 003 was found to effectively inhibit (85%) the mycelial growth of *R. solani* (Reddy et al., 2007). *P. fluorescens* 003 was found to be highly effective in controlling *R. solani* with inhibition of 58% (Reddy et al., 2010). *P. flourescence* showed highest antifungal activity against *Penicillium italicum* (94%) and was moderately effective against *Aspergillus niger* (61%) (Mushtaq et al., 2010). Isolate of *P. fluorescens* on co-inoculation with fungal pathogens showed maximum inhibition for phytopathogens of *Collectotrichum gleosporioides* (58.3%), *Alternaria brassicola* (50%), *Alternaria brassiceae* (12.5%), *Alternaria alternate* (16.66%), *Fusarium oxysporum* (14.28%) and *R. solani* (50%) (Ramyasmruthi et al., 2012). *In vitro* evaluation of antifungal activity of ethyl acetate and methanol extracts of secondary metabolites of Pf 3 at 0.1% concentration revealed that they completely inhibited the mycelial growth test pathogen (*B. fabae*) compared to hexane solvents, suggesting that the antifungal compound are completely extracted with ethyl acetate, methanol and slightly extracted with hexane. Similarly, Reddy et al. (2007) reported that the crude compounds from *P. fluorescens* isolates metabolites completely inhibited the growth of *Magnaporthe grisea, Dreschelaria oryzae*, *R. solani* and *Sarocladium oryzae* at 5%. The antifungal activity of the three solvent extracts of secondary metabolites of Pf 1, Pf 2, Pf 4, Pf 5, Pf 6, Pf 7, Pf 9, Pf 10 and Pf 11 showed that ethyl acetate extracts showed highest antifungal activity, suggesting that the antifungal inhibitory compound is better extracted with ethyl acetate than methanol and hexane. Similarly, the metabolite extracted from *P. fluorescens* with ethyl acetate was effectively inhibited (89-90%). *P. oryzae* and *R. solani* were tested at 5% concentration (Battu and Reddy, 2009). The culture filtrates obtained from *P. fluorescens* showed the inhibition of 55.2% against *Stenocarpella maydis* (Petatán-Sagahón et al., 2011). It has been observed that the filtrates obtained in logarithmic phase from the *P. fluorescens* 16 inhibited 54% of the growth of *Stenocarpella maydis* (Petatán-Sagahón et al., 2011).

Petatán-Sagahón et al. (2011) observed that the culture filtrates obtained from *Pseudomonas* spp. showed a low inhibition (5.0%) against *Stenocarpella maydis*. Maleki et al. (2010) observed that the antifungal activity of *P. fluorescence* CV6 showed higher mycelial inhibition against *Colletotrichum gloeosporioides.* The maximum inhibition of conidial germination of *Fusarium oxysporum* was brought out by 2% *P. fluorescens* (83.15 %) and the inhibition of radial mycelial growth of pathogen was effected by 2% concentration of culture filtrate of *P. fluorescens* (60.0 %) (Rajeswari and Kannabiran, 2011).

In vitro evaluation of antifungal activity of hexane extracts of secondary metabolites of Pf 8 revealed that it completely inhibited the tested pathogen *B. fabae* compared to hexane and methanol solvents, suggesting that the antifungal compound are completely extracted with hexane and slightly with ethyl acetate and methanol. *In vitro* evaluation of *Pseudomonas* spp showed antifungal activity against *Verticillium dahliae var. longisporum* as potential biocontrol agents (Berg et al., 1998).

Bioassay activity of the three solvent extracts of secondary metabolites of Pf 12 showed that methanol extracts showed highest antifungal activity, suggesting that the antifungal inhibitory compound is better extracted with methanol than ethyl acetate and hexane. Maleki et al. (2010) had reported that antifungal activity of *P. fluorescence CV6* showed the highest mycelial growth of inhibition against *Magnaporthe grisea*.

Application of bio-primed faba bean seed (Moti or ILB 938) with Pf 9 gave the maximum reduction of chocolate spot severity at 50 days after planting of faba bean, but at 70 days, the highest reduction was recorded on ILB 938 variety whereas bio-primed faba bean seed (NC 58) with Pf 10 gave the highest reduction of chocolate spot severity at 50 and 70 days after planting of faba bean. Generally, it may be related to the ability of Pf 9 and Pf 10 to stimulate the phenol and flavonoids in faba bean plant associated with increased protection and acquired immune system against chocolate spot disease (*B. fabae*) in the crop. Data clearly indicated that in untreated plants, chocolate spot infection gradually increased on leaves during growth periods and great differences were obtained among treatments of Pf 9 and Pf 10 and untreated control.

It has been showed that the bio-priming of seeds with bacterial antagonists increases the population load of the antagonist 10-fold on the seeds and thus protected the rhizosphere from the invasion of plant pathogens (Callan et al., 1990). Furthermore, the use of bio-priming seeds could be considered a safe, cheap and easily applied biocontrol method to be used against soil borne plant pathogens and physiological aspects of disease control which involves coating the seed with fungal or bacterial biocontrol agents (El-Mougy and Abdel-Kader, 2008). *P. fluorescens* strain possessing multiple mechanisms of broad spectrum antagonism and PGP activities can be explored as one among the best biocontrol agent (Ramyasmruthi et al., 2012). Maleki et al. (2010) reported that *P. fluorescence* CV6 had a broad spectrum antifungal activity against phytopathogens that can be used as an effective biological control candidate against devastating fungal pathogens that attack various plant crops. Tesfaye and Kapoor (2004 and 2007) also observed that *Trichoderma* and *Gliocladium* have greatest potential for the control of *Botrytis* corm rot (*Botrytis gladiolorum*) of *Gladiolus in vitro* and *in vivo* conditions.

To conclude, application of fungicides for disease control are largely affecting human health, normal flora and fauna, soil microorganisms and environment and also lead to the pathogenic fungi becoming very fast resistant to fungicides. For this reason, seed inoculation with *P. fluorescens* isolates as a bio-primed seed that showed antagonistic activities against *B. fabae* is an acceptable alternative to chemical fungicides application. Based on the present studies, *P. fluorescens* isolates under investigation possess a variety of promising properties which make them better biocontrol agents that are capable of producing antifungal substances and subsequent enhancement of yield of faba bean crop. The uses of *P. fluorescens* isolates Pf 9 and Pf 10 as bio-primed faba bean seed are an effective strategy for management of chocolate spot disease as well as reducing disease severity and incidence in faba bean in green house during pathogenicity test. The result of this study indicate that *P. fluorescens*, Pf 9 and *P. fluorescens* Pf 9 isolates have great contribution for control of chocolate spot disease (*B. fabae*) of faba bean *in vitro* and *in vivo* conditions.

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In vitro degradation of natural animal feed substrates by intracellular phytase producing Shiwalik Himalayan budding yeasts

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Himalayas are the natural reservoir of complex and diversified gene pool. Three Shiwalik Himalayan intracellular phytase producing budding yeasts were assayed for *in vitro* degradation of natural animal feed substrates. Phosphorus availability was found to enhance upto 70% yeast cultures during *in vitro* biodegradation of natural animal feed substrates. A direct correlation between intracellular phosphate concentration and phytase activity suggested the use of whole cell preparations in place of purified enzymes. Zymogram analysis revealed the presence of single high molecular weight isoform of the enzyme phytase. Based on 5.8S-ITS-rDNA sequencing, using ITS1 and ITS4 primers, the cultures were identified as *Candida tropicalis* (B4), *Issatchenkia orientalis* (PA4) and *Pichia gluermondii* (SS1). Indigenous *I. orientalis* strain PA4 was found superior among all the yeasts strains and therefore can be developed as successful inoculant for animal nutrition as well as environmental management under Himalayan ecosystems.

Key words: Phytase, Shiwalik Himalaya, phytase biodegradation, 5.8S-ITS rDNA, Yeast identification.

INTRODUCTION

Phosphorus (P), like nitrogen, is an essential element for all forms of life. But approximately 75 to 80% of the total P in nature is found in the fixed organic form- phytate (myo-inositol 1, 2, 3, 4, 5, 6-hexakisphosphate, IP6). The phytic acid is the primary storage form of P in plants; constitutes 3-5% of dry weight of seeds in cereals and legumes that are used as principal components of animal feeds. It acts as an anti-nutritional component in plant-derived feed; as a result they are undesirable for mono-gastric animals. The excess of P in the feed that remains unutilized is partly excreted in manure and results in pollution of ground water leading to eutrophication of freshwater bodies. Facing the problem of P deficiency in plants and animals feed, together with its pollution in areas of intensive livestock production, phytase seems destined to become increasingly important. Phytase, myo-inositol 1,2,3,4,5,6-hexakisphosphate phospho-hydrolases (EC 3.1.3.8) belongs to a sub-class of the family of histidine acid phosphatase as it can catalyze hydrolysis of phytate to inositol and orthophosphoric acid (Guilan et al., 2009).

Himalayan regions are well known for their diversified flora and fauna. Yeasts from these icy heights are well studied and characterized (Sourabh et al., 2012). The distribution of phytase is widespread among bacteria, yeast, fungi, plants, and also in animals (Mittal et al., 2012). However, negligible information is available about the phytase producing Shiwalik Himalyan Yeasts. Present study describes the phytase producing potential of the indigenous Himalayan yeast strains.

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Supplementation of yeast to animal feed as bio-inoculants can be an alternative approach to tackle P unavailability effectively because many yeast strains are already being used as single cell protein (SCP). In this perspective, *Issatchenkia orientalis* strain PA4 as an intracellular phytase producing yeast is particularly well adapted to the fluctuating temperatures of the Himalaya and could be used effectively as a low cost bioinoculant in Himalayan livestock nutrition and environmental management.

MATERIALS AND METHODS

Yeast cultures and screening for phytase production

Standard culture of *Saccharomyces cerevisiae* ATCC-9763 was procured in freeze-dried form from MTCC Chandigarh, India. Three budding yeast isolates (SS1, B4 and PA4) used in this study were obtained from departmental culture collection and revived on yeast extract peptone dextrose (YPD). Initially, the cultures were isolated from *Musa acuminata* fruit surface (B4), *Malus domestica* fruit surface (PA4) and *Sorghum bicolor* stem juice (SS1) from Pantnagar (29.00°N/79.28°E), a subtropical region of Indian Shiwalik Himalayas. The active cultures were screened qualitatively for phytase production using phytase screening medium (PSM) as described by Lambrechts et al. (1992). Sodium phytate (2 gL^{-1}) was filter sterilized and added to the sterilized medium before pouring. Yeast cultures were pin-point inoculated on MPSM plates using tooth pick and incubated at 30±1°C for 24 to 48 h. The plates were visualized for the microbial growth and the clear (halo) zone forma-tion around the colonies following the method described by Yanke et al. (1998).

Prediction of growth pattern in response to P availability

For determining growth pattern, active cultures were inoculated individually (at 5% v/v) in MPSM broth containing 0.3% KH_2PO_4 or 0.3% sodium phytate separately and incubated at 30±1°C. The samples were withdrawn periodically at an interval of 2 h, upto a period of 96 h, till the stationary phase was achieved. Yeasts growth rate were analyzed overtime according to the Gompertz equation modified by Zwietering et al (1990):

$$y = A.\exp\{-\exp[(\frac{\mu_{max}.e}{A}).(\lambda - t) + 1]\} \quad (1)$$

Where, y is O.D. value at time t (h), A represents the maximum O.D. (when t =∞), μ_{max} is the maximum specific growth rate (h^{-1}) and λ is the lag time (h).

Generation time (mean doubling time) was calculated using the following formula:

$$g = 0.693/\mu \quad (2)$$

Where, g = generation time and μ = growth rate constant.

For modeling with Gompertz equation, the means of three replicates and two repetitions were used (Tofalo et al., 2009). In all the cases, the variability coefficient of raw data (cell load as O.D.) was <5%. The data relative to the growth kinetics were subjected to student's test to identify significant differences between yeast species using "Non-linear Regression Analysis" (NLREG, USA).

Estimation of phytase activity

For crude enzyme preparation, the cultures were grown in MPSM medium at 30±1°C (120 rpm) in an incubator shaker for 96 h. Samples withdrawn periodically at an interval of 24 h, were centrifuged at 10,000 rpm (4°C) for 10 min. The culture filtrate was used as crude enzyme for determining extracellular phytase activity while cell pellet obtained as above was used for determining intracellular whole cell enzyme activity (Shimizu, 1992). The cell extracts for determining cell free intracellular phytase activity were prepared by the sonication protocol (Ruiz et al., 1999). Cell pellet were washed twice with normal saline (0.1% NaCl) and suspended in phosphate buffer (0.1 M, pH 7.0) in 1:1 ratio. Each cell suspension was sonicated on ice, at wave amplitude of 2 μm, for 6 cycles (30 s each) with 10 s interval after each cycle. PMSF was added to the final concentration of 1 mM. The sonicated suspension was centrifuged at 10,000 rpm for 10 min at 4°C. Supernatant was used as crude enzyme for quantification. The phytase activity was assayed following the method described by Bae et al. (1999). One unit of phytase activity was defined as the amount of enzyme that released 1 μmol phosphate per min at 37°C. All determinations were performed in triplicate.

Partial purification of enzyme

The sonicated suspension, prepared as above was centrifuged at 10,000 rpm (4°C) for 30 min and passed through bacterial filters. The filtrate was concentrated to 1/5 of the original volume by ultra filtration through Omega ™ polyether sulfone ultra filtration membrane capsule of 10 KDa molecular weight cutoffs (MWCO) using Minimate ™ Tangential Flow Ultra filtration system (Pall life science, USA). Protein concentration was measured by Bradford's method (Bradford, 1976) with bovine serum albumin as standard.

Electrophoresis and phytase zymogram analysis

The ultra filtered purified phytase samples were fractionated by polyacrylamide gel electrophoresis. Zymogram technique was used for phytase isoform analysis and activity bands were visualized by Native PAGE through activity staining as described earlier (Laemmli and Favre, 1970). The gel photograph was documented using gel documentation system (GelDocMEGA, UK).

Phytate biodegradation assay

The protocol for phytate biodegradation was adopted from the method described by Quan et al. (2001). Natural animal feed samples (wheat bran, rice bran, and sweat sorghum baggase) were autoclaved for 20 minat 120°C to inactivate any phytase present in them. 10 g of the autoclaved sample was suspended in 100 ml of acetate buffer (0.2 M, pH 5.5). The suspension was incubated with 1 ml of cell suspension (prepared as described in section 2.3) at 30°C on an incubator shaker at 110 rpm. The reaction was stopped at various incubation periods by adding an equal volume of 10% trichloroacetic acid. The amount of liberated inorganic phosphate was measured by taking absorbance at 700 nm.

Molecular characterization and identification of yeasts

All the three isolates *viz.* SS1, B4, and PA4 were characterized on the basis of 5.8S- ITS rRNA gene sequences. The polymerase chain reaction (PCR) amplification and sequencing of partial 5.8S-ITS rRNA gene was carried out with the standard primer set ITS1 (forward, 5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (reverse, 5' TCCTCCGCTTATTGATATGC 3') as described earlier (Bruns et al., 1991). The sequences (5.8S-ITS rRNA gene) were then analyzed by Basic Local Alignment Tool (BLAST) at NCBI database. Phylogenetic tree was constructed based on 5.8S-ITS rRNA gene sequences by using MEGA4x1.BETA2 software (N-J plot method).

RESULTS AND DISCUSSION

Qualitative screening for phytase production

All the isolates (SS1, B4 and PA4) along with standard culture of *S. cerevisiae* showed good growth on MPSM medium during plate assay. This indicates their capabilities to hydrolyze sodium phytate through phytase activity and utilize it as their sole P source. However, none of them showed the formation of any clear zone (halo zone) around the colonies, indicating thereby the intracellular location or cell surface attachment of the enzyme. Yeast have been well reported as rich genetic resources for production of extracellullar (*Schwanniomyces castellii* and *S. cerevisiae*) and intracellular (*S. cerevisiae, Cryptococcus laurentii and Candida krusei*) phytases (Man-Jin et al., 2008).

The isolate PA4 showed maximum biomass yield (1.79 g L^{-1}) that was significantly higher than the other cultures tested under P-deficient condition. Comparatively lower biomass yield for all sodium phytate grown cultures was recorded as compared to that in the presence of available P source (KH_2PO_4). Sodium phytate is a complex organic phosphorus source that is not readily available and therefore growth rate of yeast on sodium phytate depends upon its phytase producing potential. A direct correlation between growth phase and biomass yield of the cultures was observed during growth on sodium phytate (Figure 1). The isolate PA4 showing maximum growth and biomass yield on sodium phytate was found superior among all the cultures tested including standard culture (*S. cerevisiae*). A strong correlation between biomass yield and the growth phase of the cultures indicates strong growth-associated phytase production.

Growth behavior in response to P availability

Various growth parameters of the cultures *viz.* maximum logarithmic growth phase (A_{max}), growth rate constant (μ_{max}), lag phase (λ) and generation time (g) were calculated in response to P availability (Table 1). Among the four cultures, the isolate PA4 showed maximum log phase value (A_{max}, 2.35) with lowest generation time (6.30 h^{-1}), while growing at the expense of sodium phytate (Figure 2). A general decline in O.D. observed in the presence of complex organic P source, might be attributed to a slower proliferation of yeast cells due to the P stress. Similar type of growth decline due to abiotic stress in yeast has also been reported earlier (Sourabh et al., 2012). An extended lag phase (λ_{max} 14.25) under P starvation condition indicated clearly that the yeast takes longer adaptation time in the stress conditions, but once adapted, it starts growing exponentially at the expense of phytate that was hydrolyzed through the phytase activity.

Intracellular phytase activity

The intracellular enzyme activity (U mg^{-1} wet biomass) varied from 4.85 to 31.26 in whole cell and 4.43 to 30.97 for cell free preparations at 96 and 48 h of incubation period, respectively (Table 2). Slightly higher whole cell intracellular enzyme activity as compared to the cell free activity might be attributed to the mechanical loss of the enzyme during the enzyme preparation procedure especially sonication (Ruiz et al., 1999). The isolate PA4 showed maximum phytase activity (31.26 U mg^{-1} wet biomass) at 48 h of incubation period. A slight difference in the whole cell and cell free intracellular enzyme activities observed during present investigation reveals the better use of whole cell preparations in place of purified enzymes. The cytoplasmic phosphate content of phytate grown cells increased gradually up to 72 h and then it decreased invariably in all the cases and varied from 69.75 (SS1) to 189.00 (PA4) µg ml^{-1} at 24 and 72 h, respectively. It is because of the better availability of phosphate in culture medium due to solubilization of sodium phytate through phytase activity of yeast cells. A direct correlation was thus observed between the phytase activity and increasing P-content in cells with respect to time (Figure 3). However, a decrease in P content of the cells after 72 h might be correlated with higher rate of phosphate requirement and its uptake by the cells as compared to its availability in the medium due to poor phytase activity. Inhibition of phytase activity upon prolonged incubation might be due to the inhibitory effects of some intermediates produced during growth on phytate and/or possibly cells might have entered into the stationary phase, switching off expression of the enzyme (Hatzack et al., 2000).

In vitro biodegradation assay

In vitro biodegradation system (wheat bran, rice bran and sweet sorghum bagasse) consisted of autoclaved samples in acetate buffer (0.2M, pH 5.0) inoculated with cell suspensions and phytate hydrolysis was allowed to occur at 37°C for four days to simulate the conditions of the digestive tract; phosphate released in various samples was measured (Figure 4). Once biodegradation began, the phytate in the samples was gradually hydrolyzed and phosphate liberated. The increasing phosphate concentration with respect to incubation period in all the feed samples indicates clearly the potentiality of all the cultures to hydrolyze phytate through phytase activity. Among the four samples evaluated, maximum and significantly higher phosphate release (0.71 mg g^{-1} of sample) was shown by the isolate PA4 in rice bran. Since yeast cultures are used as starter cultures for leavening of bread dough, therefore, the isolates having intracellular phytase activity at longer time can have a better application in bread making with long leavening time (Bohn et al., 2008). Moreover, as reported earlier also, the exogenous phytase is not primerly required rather whole yeast cells can also be use for phytate degradation during bread making, unless whole grains are added to the mixture (Bohn et al., 2008).

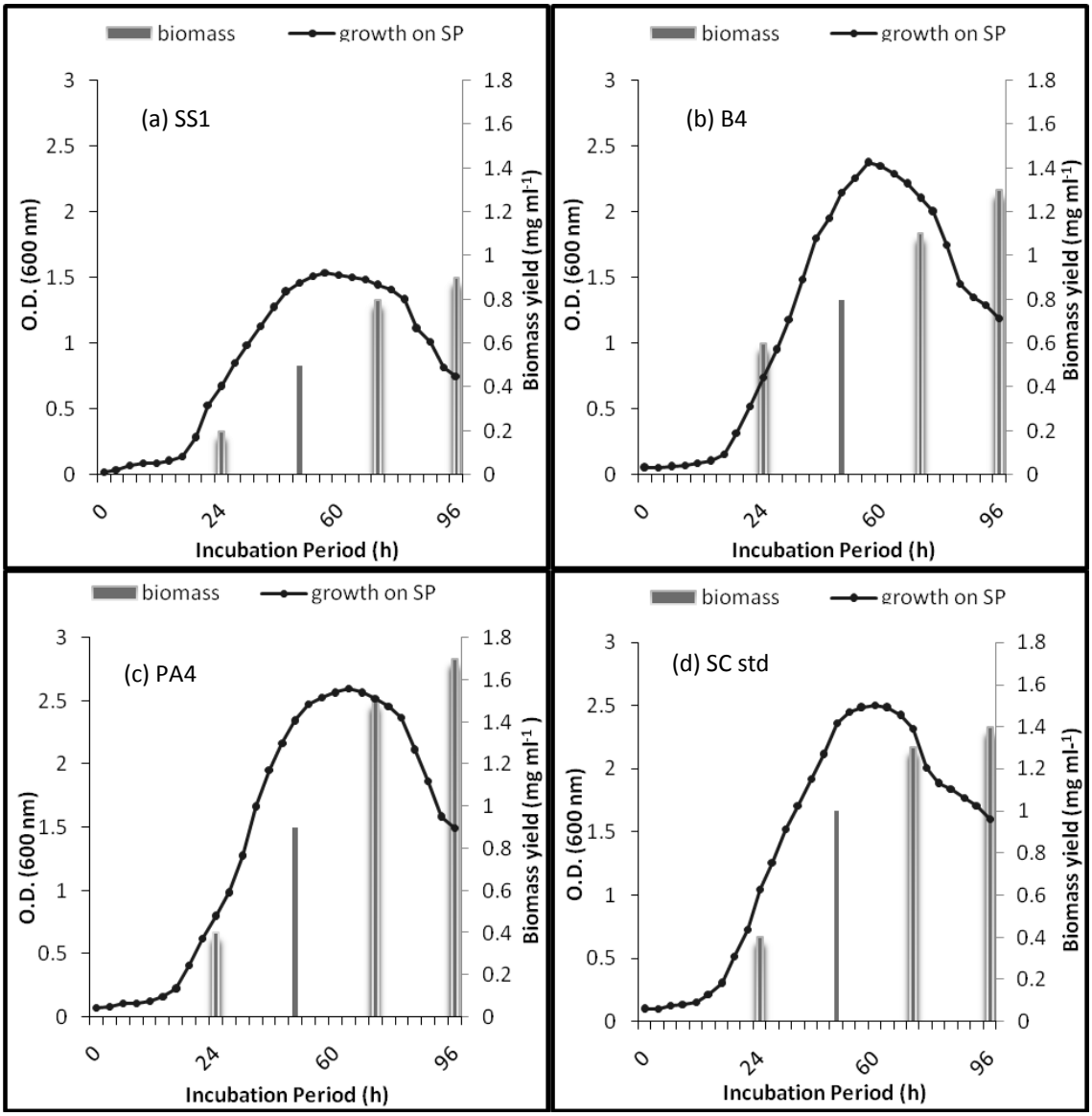


Figure 1. Correlation between biomass yield and growth of SS1 (a); B4 (b); PA4 (c) and SC-std (*S. cerevisiae)* (d).

Table 1. Growth parameters of yeast isolates with respect to phosphorus availability.

P source	Culture/ Isolate	Growth parameter			
		A_{max} (O.D.)	μ_{max} (h^{-1})	λ (h)	Generation time (g)
Unavailable P (sodium phytate)	SS1	1.35±0.06	0.07±0.01	11.52±0.11	9.90
	B4	1.73±0.08	0.09±0.03	14.42±0.13	7.70
	PA4	2.35±0.06	0.11±0.02	14.27±0.13	6.30
	SC std	2.31±0.03	0.10±0.03	13.44±0.15	6.93
Available P (KH_2PO_4)	SS1	3.08±0.02	0.10±0.01	5.92±0.09	6.93
	B4	3.22±0.07	0.11±0.01	4.13±0.12	6.30
	PA4	3.31±0.06	0.12±0.03	8.05±0.11	5.77
	SC std	3.31±0.03	0.13±0.02	7.95±0.13	5.30

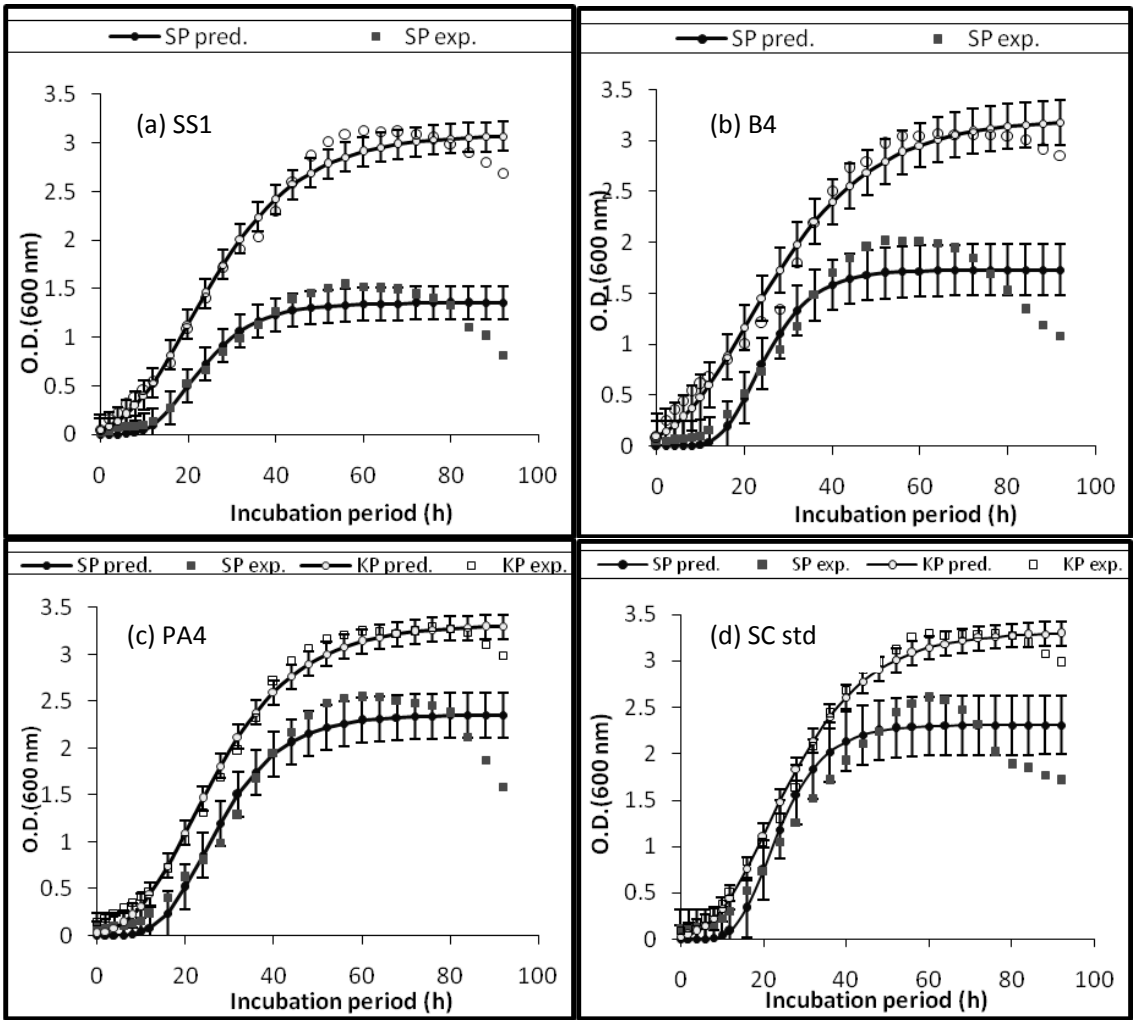


Figure 2. Growth behavior of various yeast cultures under P-stress (sodium phytate) isolates and SC-std (*S. cerevisiae,* d) in MPSM medium and P-sufficient (KH_2PO_4) conditions in MPSM broth. SP: Sodium Phytate; KP: KH_2PO_4; Exp: Experimental; Pred: Predicted.

Table 2. Intracellular (whole cell and cell free) phytase enzyme activities of various cultures of yeast grown on sodium phytate mineral medium (MPSM).

Culture/ Isolate	Intracellular Phytase activity (U mg^{-1} wet weight of biomass) at different time intervals (h)							
	Whole cell activity				Cell free activity			
	24	48	72	96	24	48	72	96
SS1	10.00	20.24	10.92	6.52	9.42	18.10	10.68	6.14
B4	16.68	23.11	15.45	6.55	13.61	22.63	15.40	5.94
PA4	15.32	31.26	13.00	4.85	13.27	30.97	12.52	4.44
S. cerevisiae (SC std)	12.60	23.14	13.13	5.03	12.35	22.55	12.97	4.43
CD at 5% a (Isolates)	0.12401 ; b (Interval) 0.13125; a*b (Interaction) 0.25526							

Zymogram analysis

The partially purified intracellular protein samples when analyzed for phytase isoforms by activity staining through native PAGE, only one sharp band could be visualized in each test sample (Figure 5a). Results suggest that the molecular mass of native protein from different cultures fall between 100-135 KDa with different band intensities.

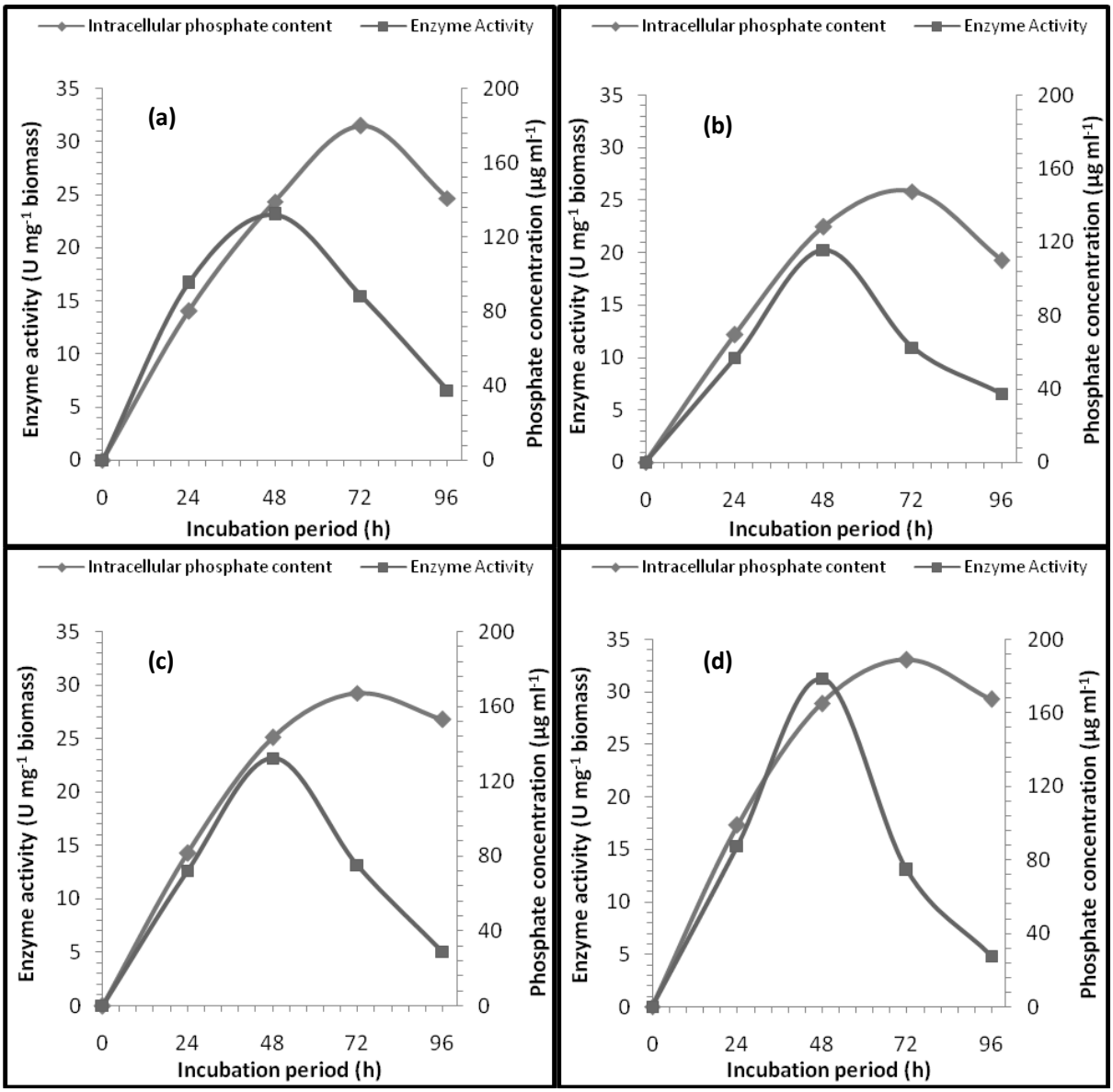


Figure 3. Intracellular phosphate concentration with respect to phytase production in MPSM grown yeast isolates: SS1 (a); B4 (b); PA4 (c) and SC-std (S*. cerevisiae)* (d).

The SS1 phytase showed a very faint band in comparison to others, while the intensities of B4 and PA4 phytase bands were higher than *S. cerevisiae* phytase band on zymogram. Single band pattern on zymogram shows that all the test strains of yeast produce only one isozyme for the phytase enzyme. The molecular weight of several yeast phytases such as *Debaryomyces castelli* and *Schwanniomyces castellii* have also been reported to be in the range of 72 to 125 KDa (Wyss et al., 1999).

Molecular characterization

PCR products showed a high length variation in this region for different yeast cultures, approximately 850 bp for *S. cerevisiae* (SC-std, lane-1), 600 bp for the isolate PA4 (lane-2), 650 bp for the isolate B4 (lane-3) and 700 bp for the isolate SS1 (lane-4) (Figure 5b). Variable length of the 5.8S-ITS rDNA PCR product obtained for different cultures indicates clearly that they belong to different yeast genera; because, PCR products from different strains of the same species and from different species of the same genus should have identical or very similar molecular size (Sourabh et al., 2012). Variable size of the PCR product of 5.8S-ITS rRNA gene for different yeast cultures belonging to different genera and species has also been reported for different yeast genera *viz Candida*, *Pichia,* etc (Guillaman et al., 1998).

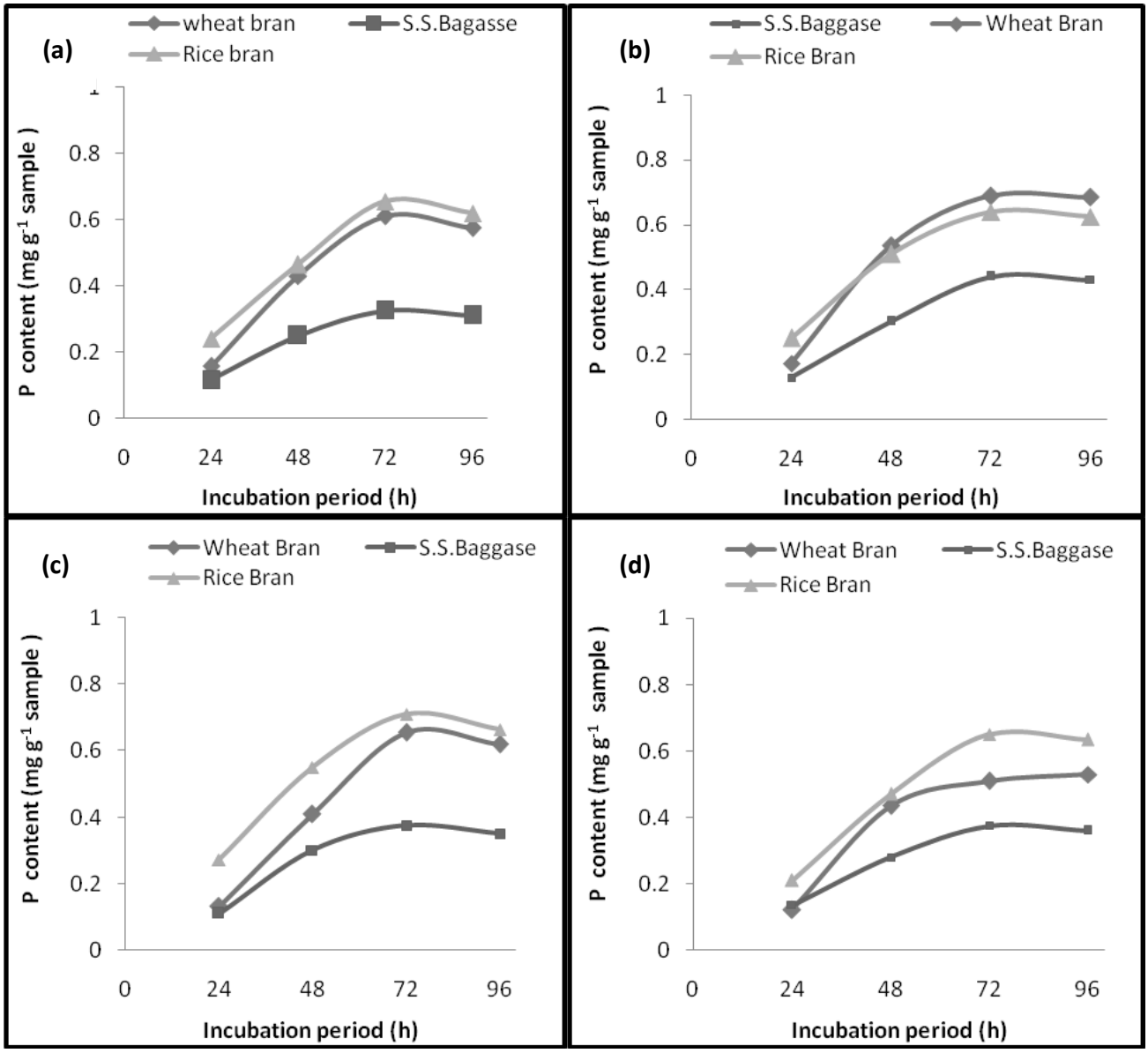


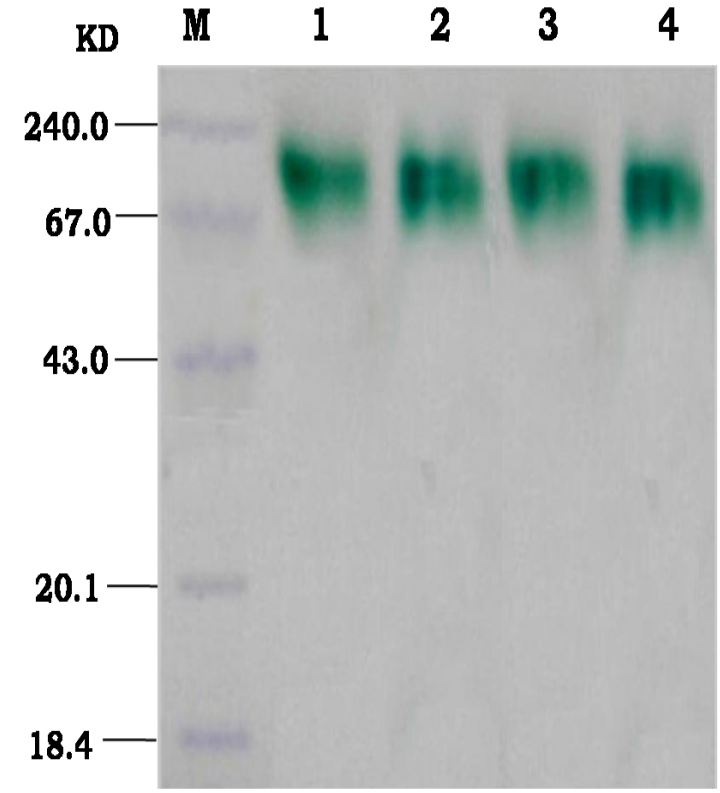
Figure 4. Phosphate released upon biodegradation of phytate in natural feed substrates by various yeast cultures: SS1 (a); B4 (b); PA4 (c); and SC-std (S. *cerevisiae*) (d).

Identification of yeast isolates

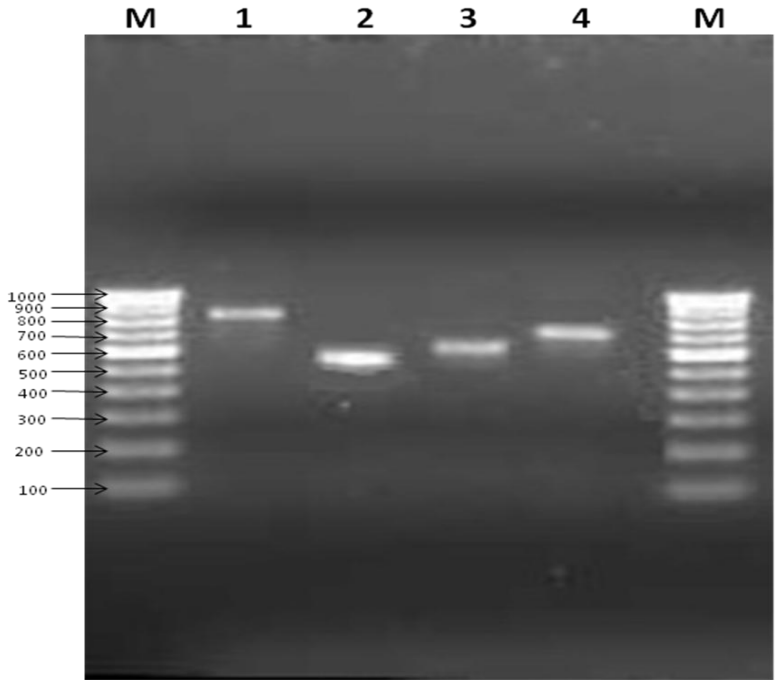
NCBI-BLAST sequence similarity search based on 5.8S-ITS rRNA gene sequence identified the isolates B4, SS1 and PA4 as *C. tropicalis* (EF190223.1), *P. guilliermondii* (DQ088676.1) and *I. orientalis* (FJ697171.1), respectively (Table 3). Phylogenetic tree was constructed based on 5.8S-ITS rRNA gene sequence alignment using MEGA4 software (N-J plot method) (Figure 6).

Conclusion

The study reveals a vast potential of Western Indian Himalayan budding yeast belonging to diverse genera, *Candida, Pichia* and *Issatchenkia* for organic phosphorus solubilization. The high intracellular phytase activity along with high protein content of various isolates further emphasizes the use of intact cells as feed additive in place of purified enzymes for enhancing mineral bio-availability and thereby minimizing the cost of ensilage production. *I. orientalis* strain PA4 showing maximum intracellular phytase activity and phosphorus bioavailability in animal feed substrates indicates future prospects for this culture to be developed as a bioinoculant for animal feeds and various other industrial applications.



(a)



(b)

Figure 5. Electrophoretic analysis. **(a)** isoforms by Native PAGE through activity staining: Lane M, protein marker; lane 1, SC,std (*S. cerevisiae*); lane 2, SS1; Lane 3, B4; lane 4, PA4. **(b)** PCR amplified 5.8S,ITS rRNA gene products of various yeast cultures: lane M,100 bp DNA ladder; lane 1, SC std; lane 2, PA4; lane 3, B4; lane 4, SS1.

Table 3. List of 5.8S rRNA gene sequences from gen bank data base along with the isolate SS1, B4,PA4 and SC-std. Sequence comparison of the gene by EMBOSS Alignment (http://www.ebi.ac.uk/EMBOSS/align/).

Gen bank accession number	Origin	Gene name	Identity (%)			E value
			SS1	B4	PA4	
EF190223.1	*Candida tropicalis*	5.8S rRNA	63.3	99	50.7	0
DQ088676.1	*Pichia guilliermondii*	5.8S rRNA	99.3	68.9	41	0
AB369918.1	*Issatchenkia orientalis*	5.8S rRNA	55.3	55.6	99	0
AB533542.1	*Saccharomy cescereviseae*	5.8S rRNA	46.1	48.2	34.4	0
JF920159	SS1	5.8S rRNA	100	69.4	41	-
JF300164	B4	5.8S rRNA	69.4	100	57.6	-
JF920160	PA4	5.8S rRNA	41	57.6	100	-

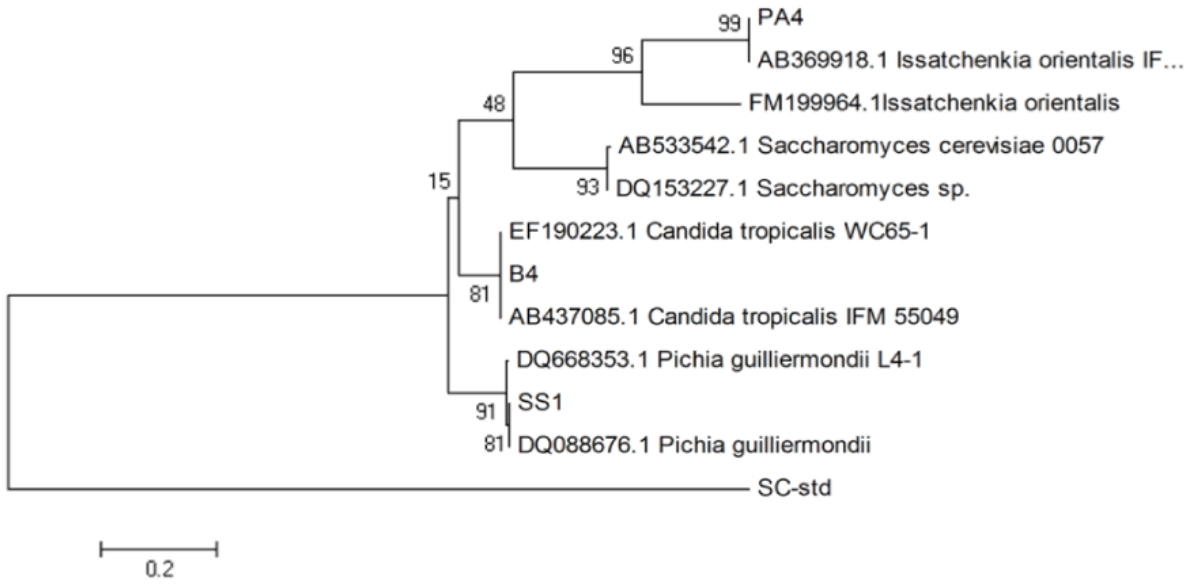


Figure 6. Phylogenetic tree showing evolutionary relatedness of the yeast isolates with closely related yeast species.

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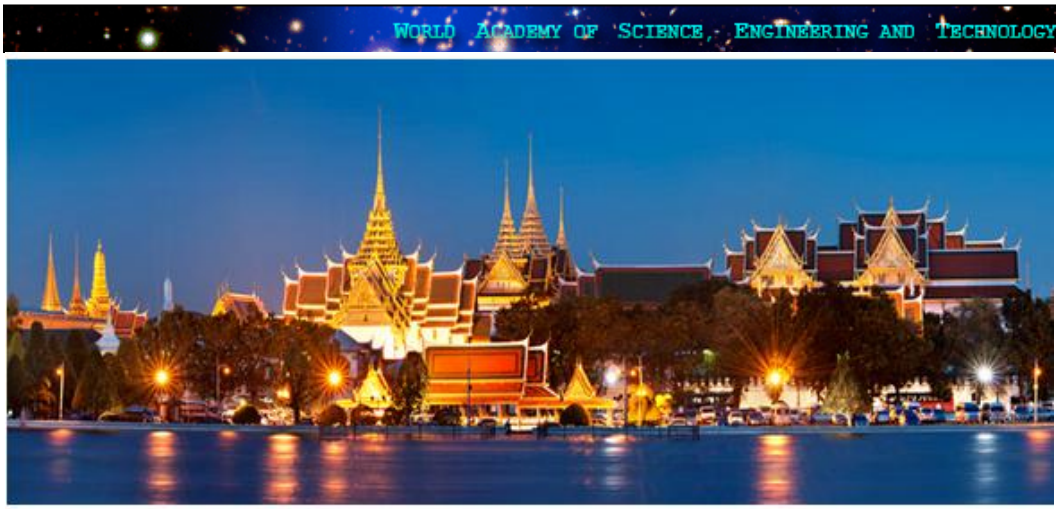

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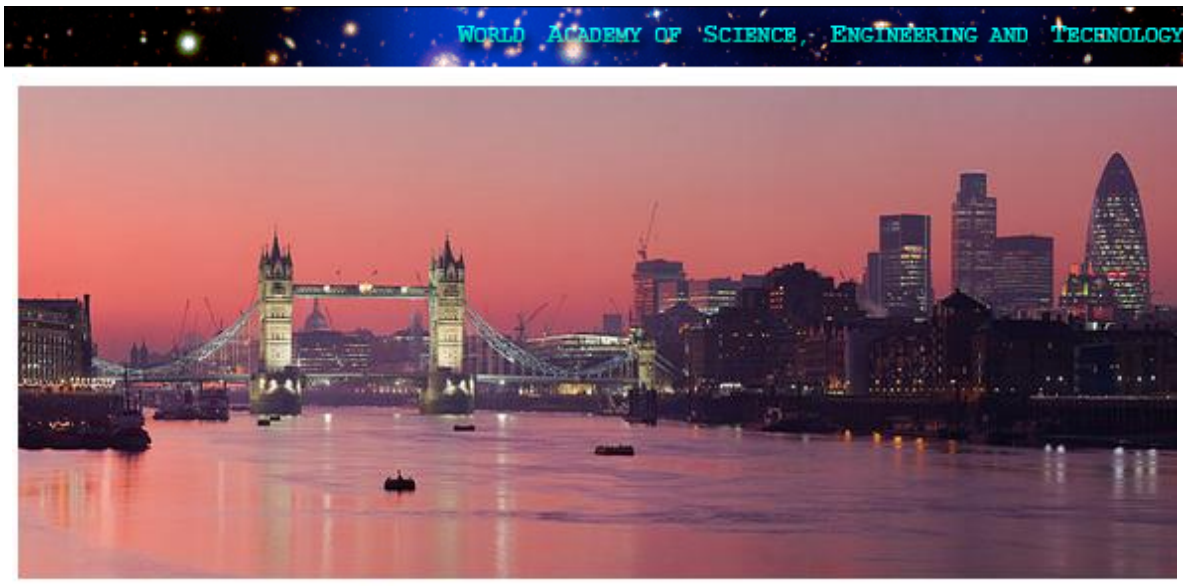

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