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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

Chemical composition and antifungal activity of leaf powders and extracts of *Byrsonima crassifolia* (L.) Kunth, *Ocimum basilicum* L., *Persea americana* Miller., *Psidium guajava* L. and *Spondias purpurea* L. from Morelos, México

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Natural products from plants have great potential as novel fungicide sources for controlling pathogenic fungi. The aims of this study were to examine the chemical composition of the leave powders and extract (Hexane, methanol and water) of Mexican plants and to test *in vitro*, their efficacy as antifungal potential against *Fusarium oxysporum* f. sp. *gladioli*. All the species showed antifungal activity in the methanol extract. Chemical analysis of leave powders and extracts of *Byrsonima crassifolia*, *Ocimum basilicum*, *Persea americana*, *Psidium guajava* and *Spondias purpurea* by gas chromatography and spectrometry of masses (CG-MS) analysis showed that 89 volatile compounds were present. Leave powders and extracts contained the following: sesquiterpenes (46.06%), fatty acids (23.6%), diterpenes (14.6%), phenolic compound (11.23%) and monoterpenes (4.7%). Sesquiterpenes were not detected in *B. crassifolia.* The powders and leaf of extract, at 5% (hexane and methanol) revealed remarkable antifungal effect in the growth inhibition of *Fusarium oxysporum* f.sp. gladioli with a range of 15 to 67%. The high chemical diversity of the analyzed plant species results in different effects on the development of the fungus.

Key words: Gladiolus, botanic extracts, pathogenic fungi, growth inhibition.

INTRODUCTION

It has been proven that medicinal plants exhibit antifungal and antibacterial activities*. Byrsonima crassifolia, Ocimum basilicum*, *Persea americana* and *Psidium guajava*are are widely used in Mexico to treat gastrointestinal and respiratory disturbances and are used as anti-inflammatory medicines (Aguilar et al., 1996). Plant diseases caused by plant pathogenic fungi are among the most important factors that limit plant ornamental production in México.

The continuous application of fungicide chemicals will lead to the destruction of the ecosystem and may result in the outbreaks of disease from new strains of fungi that are difficult to control. To minimize the side effects of chemical application, the antifungal activity of plant extracts have been utilized. Several studies showed the importance of natural chemicals as a possible source of non-phytotoxic, systemic and easily biodegradable alternatives (Al-Mughrabi et al., 2001; Bautista-Baños et al., 2002; Garduño-Pizaña et al., 2010)

Gladiolus producers in México are faced with several phytosanitary problems, both in the plant and in the corms. Standing out among the major diseases is fusarium disease, the causal agent which is *Fusarium oxysporum* f. sp. *gladioli*. It is considered the most destructive and widely distributed disease in most gladiolus-producing countries in the world. *F. oxysporum* f. sp. g*ladioli* is an important pathogen that can reduce the production of gladiolus corms and flowers, inflicting severe economic losses on producers. The specific objectives of the present work were to determine the *in vitro* antifungal activity of 5 Mexican plant species on mycelial growth of *F. oxysporum* f. sp. *gladioli* and to identify the volatile compounds.

MATERIALS AND METHODS

Plant material

This study was carried out in the Biotic Products Development Center in Yautepec, State of Morelos, México in January-December, 2010. Fifty different plant species corresponding to various plant families and including the species below which are shown to have high fungistatic or antiparasitic activity over human diseases (Aguilar et al., 1996) were evaluated for their antifungal activity: *Byrsonima crassifolia* (L.) Kunth, *Ocimum basilicum* L., *Persea americana* Miller., *Psidium guajava* L., *Spondias purpurea* L. Except for *Ocimum basilicum,* mature leaves were harvested from trees ten years old grown at Amacuzac, Tetecala and Yautepec within the state of Morelos, in these sampling sites, the climate is either tempered, wet warm or wet tropical with annual precipitancy of 754.6 to 1187 mm. Once harvested, leaves were sorted, discarding damaged or diseased material. Plant material was dipped in 1% sodium hypochlorite, rinsed with distilled water, air-dried, macerated with the aid of a blender and a grinder and stored in amber bottles until further use.

Test microorganism

Fusarium oxysporum f.sp. *gladioli* were isolated from gladiolus corm rots at Cuautla, Morelos, México and previously was morphologically and molecularly characterized. The isolates were maintained on Potato Dextrose-Agar (PDA) in Petri plates at temperature of 26°C. To maintain pathogenicity of the fungus, periodic inoculations and reisolations from infected corms were carried out.

Preparation of powders and extracts

To evaluate leave powders, 20 mg ml^{-1} (w/v) of the macerated material was added to plates containing PDA. Dry powders of leaves (50 g) were mixed with hexane, methanol and water (500 ml) one after the other, for 24 h in each solvent system at room temperature according to Reyes-Chilpa et al. (1998). After each extraction step, the leave extracts were filtered and concentrated in a rotary evaporator (Buchi R-114, LabortechnikFlawil, Switzerland) and then stored at 4°C in amber bottles until use. Plant powders and aqueous extracts were added to PDA, autoclaved (15 lb/cm^2, 15 min) and poured into Petri plates (100 x 15 mm) (Bautista-Baños et al., 2000). The hexane and methanol extracts were added to PDA after sterilization media and poured into Petri plates (60 x 15 mm). A five mm agar disc of a 9 days old colony of the pathogen was placed at the center of each plate and incubated at 25°C for 8 to 14 days in the dark. The colony diameter was recorded for each treatment until fungal colonies in the control treatment reached the edge of the plate. The percentage of inhibition was I(%) = [(C-T) /C] x 100 where C represents the growth in the non-amended control and T in the treatment. There were six replicates for each treatment. Control Petri plates contained only PDA and solvent with PDA. Control of PDA was used for comparisons with treated plates. The experiment was repeated twice.

Gas chromatography and spectrometry of masses (CG-MS)

In Center of Chemical Investigations of the Autonomous University of the State of Morelos (C.I.Q. - U.A.E.M), GC-MS analyses were performed using an Agilent 6890 series GC systems (Agilent Technologies, Santa Clara, CA) coupled to a quadrupole mass spectrometer (model 5973) equipped with an HP5-MS with stationary phase of 5% phenyl methyl siloxane capillary column (25 m x 0.20 mm x 0.33 µm film thickness). For GC-MS, detection electron ionisation with ionisation energy of 70 Ev was used a scan range of 30-550 atomic mass units. Helium was the carrier gas, at a flow rate of 1 ml/min with a temperature program starting at 40°C for two minutes followed by an increase of 10°C/min until the temperature of 260°C and was maintained for 20 min. For the case of the extracts, a solution of 5 mg in 0.5 ml of respective extract was prepared. In the case of vegetal powders, before injecting itself to the chromatograph, they were entered to the headspace with a temperature of 100°C, later to the loop to 120°C and then 130°C was maintained. The components were identified by matching their recorded mass spectra with the data bank mass spectra (NIST-MS Version 1.7a) and by comparing their retention indices relative with literature values.

Statistical analysis

The treatments using powders and botanic extracts were arranged in a completely randomized design with six repetitions. Standard analysis of variance (ANOVA) were used to determine the effects of plant extracts on mycelial growth. Treatment means were compared using the least significant difference (LSD) multiple range test ($p = 0.05$). All the analyses were made in Stat Sigma version 3.5 (SystatSoftwereErkrath, Germany).

RESULTS

Antifungal activity

The leave powders of *B. crassifolia*, *P. guajava*, *S.*

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Table 1. Effect of powders and extracts of five Mexican plant on mycelial growth (mm) of *F. oxysporum* f.sp. *gladioli*.

Plant species	Powders	Aqueous extract	Methanol extract	Hexane extract
Control	85 ±2.2 (0.0) a	85±0 (0.0) a	50.0±0(0.0)a	49±0.9 (0.0)a
Solvent			47.8±1.8(4.3)ab^{ns}	49±0.5 (6.2)
Byrsonima crassifolia	62.7±2.6(26.1)c***	62.5±6.8 (26.5) bcd**	26.2±2.9(47.7) e***	50±1.1 (0.0)
Ocimumbasilicum	85±4.7 (0.0)	59.0±15.4 (30.6) cd***	28.7±3.2(42.7) cd***	29.1±4.4 (39.2)cd***
Persea americana	85±0 (0.0)	74.7±8.4 (12.1) ab^{ns}	28.3±3.2(43.3) de***	29.8±5.6 (40.6)d***
Psidiumguajava	73.6±1.5(13.2)b***	75.4±2.7 (11.3) ab^{ns}	21.4±4.3(57.2) f***	41.7±1.4 (15.0)b***
Spondias purpurea	77.4±5.8(8.7) b**	80.5±6.9 (5.3) a^{ns}	16.5± 1.4(67.0) g***	30±0.9 (38.8)c***
ANOVA	F:23.66 gl:3,20	F:9.50 gl:6,64	F:118.88 gl:7, 84	F:266.42 gl:5,28

Means followed by different letter(s) in each column are significantly different by LSD test at P≤ 0.05. Values in parenthesis indicate mycelial inhibition (%). *** P≤ 0.001 ** P≤ 0.05 ^{ns} Not significantly. Means without letter were not compared with the control and the rest of the treatments because was same that control.

purpure resulted in the highest mycelial growth (62.7-77.4 mm) when compared with methanol and hexane extracts. The aqueous extracts of *B. crassifolia* and *O. basilicum* displayed similar mycelial growth (59.0-62.5 mm). Methanol extracts of all the botanic species displayed lowest mycelia growth of 16.5 to 28.7 mm. Hexane extracts of *O. basilicum, P. americana, P. guajava* and *S. purpurea* showed minor mycelial growth when compared with the control (29.8-41.7 mm). The different powders and extracts inhibited significantly (p≤0.001) the mycelial growth. Mycelial inhibition was of 26.5 to 30.6% with leaf aqueous extracts. The methanol extracts inhibited the mycelial growth of this fungus more than 40%. The highest mycelial inhibition was obtained with methanol extracts of *S. purpurea* and *P. guajava* (57.2 and 67% respectively) (Table 1).

Chemical composition

Eighty nine compounds were identified in powders and extracts by chromatographic analysis, that belong to 4 groups of compounds which include sesquiterpenes (46.06%), fatty acids (23.6%), diterpenes (14.6%), phenolic compound (11.23 %) and monoterpenes (4.7%). Most of the identified compounds were soluble in methanol and hexane. The detected volatile compounds had mol.wt. between 126 to 416 MW. The sesquiterpenes, diterpenes and fatty acids were present in most of the species. The major constituents in the methanol extract of *B. crassifolia* were nonacosane (28.14%), gallic acid (20.90%), palmitic acid (12.0%), linoleic acid (10.18%) and γ-sitosterol (8.38%). In aqueous extract, quinic acid (32.45%) and oleic acid (67.55%) and 10 minors constituents were found. The sesquiterpenes were not detected in *B. crassifolia.* On the contrary, the monoterpenes and sesquiterpenes were the main compounds detected in the species of *O. basilicum*, where the major constituents in hexane extract were linalool (22.3%), methyl chavicol (8.1%), T-cadinol (6.06%), Fi 1,6 diene (7.81%) and in the aqueous extract γ-sitosterol (46.59%) and α-amyrin (23.09%) and 27other minor constituents . In methanol extract of *P. Americana* were found a mixture of the following major constituents: γ-sitosterol (35.97%), methyl chavicol (26.99%), quinic acid (9.10%) and in hexane extract 3-pentadecil phenol (34.0%) and palmitone (27.0%) and 13 other minors constituents. In powders and methanol extract of *P. guajava* were detected a mix-ture of 3 major compounds β-caryophyllene (27.51-17.16%), β-bisabolene (23.28%) and α-curcumene (15.86-6.8%) and 25 other minor constituents. Powders of *S. purpurea* contained a mixture of 3 major com-pounds: α-copaene (30.4%), β-cadinene (52.0%) and β-cadinol (17.6%), and in hexane extract estragol (33.85%) and methanol extract heptadecenilphenol (35.62%) and 25 other minor constituents were seen (Table 2).

DISCUSSION

The fungistatic effects of powders, and methanol and aqueous extracts of *B. crassifolia* was observed on *F. oxysporum* f. sp. *gladioli*. In the methanol extracts, the major compound was linoleic acid, this fatty acid has been reported to have antimicrobial properties (Santoyo et al., 2006). Powders, methanol and hexane extract of *P. guajava* significantly reduced *Fusarium* mycelial growth. The methanol extracts contained greater number of compounds mainly sesquiterpenes and fatty acids, groups considered to have antifungal activity (Chang et al., 2008; Liu et al., 2008). Methanol extract and powders of *S. purpurea* inhibited mycelial growth of *F. oxysporum* f. sp. *gladioli*. These extracts are characterized by the presence of several terpenes, fatty acids and phenolic compounds which has antimicrobial activity (Deans et al., 1995). With extracts of leaf of this species, Bautista-Baños et al. (2000a) reported inhibition in the germination of *Rhizopus stolonifer*. Hexane and methanol extract of *O. basilicum* inhibited the mycelial growth with 40.6 and42.7%, respectively as compared to the control, and it

Table 2. Percentage composition of volatile compounds of hexane, methanol, aqueous extracts and powders of five Mexican plants.

Compounds	T (min)	MW	Methanol (%)	Hexane (%)	Aqueous (%)	Powders (%)
Gallicacid	-	126	20.9 B.c.		-	-
β-Pinene	8.32	136		0.46 S.p.		
Estragol (Methylchavicol)	-	148	26.99 P.a. 3.7 O.b.	33.85 S.p. 8.10 O.b.		
4 Vinil guayacol	-	150			1.38 O.b.	
Methyleugenol	-	178		1.10 S.p.		
Quinicacid	-	190	5.44 B.c. 9.18 P.a.		32.45 B.c.	
Eucalyptol	9.30	154	0.2 O.b.	1.12 O.b.		2.21 P.g.
Linalool	10.61	154	9.2 O.b.	22.3 O.b.		
α-Zingiberene	14.22	204				1.17 P.g.
β-Himachalene	14.50	204				4.13 P.g.
α-Copaene	14.66	204	1.83 P.g.	1.69 S.p.		30.4 S.p. 4.61 P.g.
β-Elemene	14.92	204	1.2 O.b.	0.70 O.b.		
α-Acoradiene	15.10	204				2.57 P.g.
α-Longipinene	15.15	204	1.14 P.g.			
β-Cariofilene	15.37	204	0.2 O.b. 5.82 P.a. 17.16 P.g.	11.0 S.p.		27.51 P.g.
α-Himachalene	15.50	204				1.99 P.g.
α-Guaiene	15.52	204	0.3 O.b.			
β-Bisabolene	15.60	204				23.28 P.g.
Aromandrene	15.63	204	4.57 P.g.	2.18 S.p.		5.90 P.g.
α-Humulene	15.78	204	1.1 O.b. 2.02 P.g.	1.40 O.b. 1.09 S.p.		3.33 P.g
β-Sesquifelandrene	15.80	204				7.44 P.g.
α-Curcumene	16.00	202	6.08 P.g.			15.86 P.g.
α- Cubenene	16.12	204	2.8 O.b.	2.78 O.b.		
β-Eudesma (β-selinene)	16.28	204	5.72 P.g.			
γ-Elemene	16.37	204	0.7 O.b.			
α- Bulnesene	16.44	204	0.5 O.b.	0.84 O.b.		
γ-Muurolene	16.53	204		1.86 O.b. 0.4 P.a.		
β-Cadinene	16.58	204	0.21 S.p.	0.4 P.a. 6.47 S.p.		52.0 S.p.
β-Cadinol	16.59	204				17.6 S.p.
γ-Cadinol	16.59	222	1.9 O.b.			
Farnesol	17.06	220		6.71 S.p.		
Nerolidol	17.08	222	3.90 P.a. 1.82 P.g.			
Trans-β-caryophyllene oxide	17.50	220	5.70 P.g. 3.15 P.a.	2.26 S.p.		
Espatulenol	17.52	220	0.5 O.b.	2.20 O.b.		
Cadine-1(10)- ene (Cubenol)	17.90	>220	0.5 O.b.			
T-Cadinol	18.17	204	2.3 O.b.	6.06 O.b.		
Ledol (Viridiflorol)	18.47	222	7.89 P.g.			
α-bisabolol	18.66	204	2.23 P.g.			
3-O-methyl-D-glucose	19.20	194			18.09 O.b.	

Table 2. Contd

Myristicacid (tetradecanoicacid)	19.50	228	0.63 B.c.		
Fit 1,6 diene	20.08	<300	2.6 O.b. 3.29 B.c. 2.34 P.a. 7.79 P.g. 2.60 S.p.	7.81 O.b. 2.10 P.a. 1.39 S.p.	
Fit 2, 6 diene	20.54	<300	0.8 O.b. 0.76 B.c.	3.55 O.b.	
Methylesterpalmíticacid	20.98	270	0.9 O.b. 4.41 S.p.		
Hexadecanoicacid (palmíticacid)	21.59	256	17.5 O.b. 10.16 S.p. 6.96 P.a. 12.00 B.c.	6.00 O.b. 5.20 S.p. 7.4 P.a.	3.93 O.b.
Linoleicacidester	22.77	292	1.13 S.p.		
Isophyitol	22.91	296	5.7 O.b. 3.55 B.c. 1.25 P.a. 1.11 P.g.	3.6 P.a. 2.98 S.p.	
Linoleicacid	23.31	280	5.21 S.P. 8.89 S.p. 17.30 P.g. 4.53 P.a. 10.10 B.c.	4.90 O.b. 10.79 S.p.	
Oleicacid	23.33	282			6.90 O.b. 67.55 B.c.
9,12,15-octadecatrienoic acid, methylester	23.33	292	1.5 O.b.		10.3 P.a.
Methylesterα-linoleicacid	23.36	292	33.9 O.b. 3.20 S.p.		
Estearicacid	23.49	284	2.71 B.c. 1.04 S.p.		
Phytol	23.81	296	1.6 O.b.		
Estearilamide	25.52	-	2.44 P.g.		
Eicocenamide	25.52	281	4.6 O.b.		
Palmitone	25.69	450		27.00 P.a.	
Fenol 3-pentadecil	27.16	304		34.00 P.a. 3.60 S.p.	
Heptacosane	30.21	380		1.12 O.b.	
Heptadecadienilphenol	30.63	204	8.88 S.p.		
Heptadecenilphenol	30.90	330	35.62 S.p.		
Condrilaesterol	32.46	412		12.84 S.p.	
γ-Sitosterol	32.85	414	8.38 B.c. 35.97 P.a.		46.59 O.b.
Erucilamide (Dococenamide)	32.90	337	3.7 O.b. 3.72 P.g. 2.87 S.p.		
Escualene	33.75	410	1.9 O.b. 4.04 B.c. 3.64 P.g. 1.55 S.p.	2.35 O.b. 2.30 P.a.	

Table 2. Contd

Nonacosane	36.08	408	28.14 B.c.	2.78 O.b.	
δ-tocoferol	38.98	402	9.51 S.p.	9.30 P.a.	
α- amyrin	39.18	426			23.09 O.b.
β-tocoferol	43.90	416	1.12 S.p.	3.00 P.a.	
Hentriacontane	45.05	-		10.0 O.b.	

B.c. = *Byrsonima crassifolia*
O.b.= *Ocimum basilicum*
P.a. = *Persea Americana*
P.g. = *Psidium guajava*
S.p. = *Spondias purpurea*

was found that the compound linalool was present at a percentage of 22, 26 and 9.2% respectively, Shatar et al. (2007) and Raseetha et al. (2009) reported fungicidal activity for this compound. Barrera et al. (2009) reported that linalool inhibited the mycelial growth of *F. oxysporum* f. sp. *gladioli.* The activity obtained from *O. basilicum* in this work, differs from the report of Adigüzel et al. (2005) who did not obtain fungicidal activity with methanol, hexane and ethanolic extracts of basil on the studied fungi, including *F. oxysporum*.

In most of the extracts and powders, we identified different compounds active on different fungi and *Fusarium.* These compounds are β-caryophyllene (present in *O. basilicum, P. american, P. guajava* and *S. purpurea*), T-cadinol (*O. basilicum*) and β-pinene (*S. purpurea*) which have a strong activity against *F. oxysporum* (Cakir et al., 2004; Chang et al., 2008). Perez et al. (1999) and Costa et al. (2000) reported activity of β-caryophyllene against *Bacillus* sp., *Candida albicans*, *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus.* On the other hand, Chang et al. (2008) reported that β-pinene inhibited the mycelial growth of *C. gloeosporioides* at a concentration of 200 µg/ml. This compound was in the hexane extract of *S. purpurea* (0.46%). Oxenham et al. (2005) also reported inhibition of *Botrytis fabae* by eucalyptol, estragol and β-caryophyllene. Eucalyptol was identified in the extracts of *O. basilicum* and *P. guajava* and estragol in the extracts of *O. basilicum* and *P. american.* Randrianarivelo et al. (2009) evaluated the effect of eucalyptol and observed that the growth inhibiting concentration of *F. oxysporum* is high (2.93 mg/ml). Cakir et al. (2004) reported that β-caryophyllene, caryophyllene, T-muurolol and γ-cadinol, have activity against *R. solani* and *F. oxysporum*. This is consistent with the observations of Chang et al. (2008), who report that the T-muurolol and γ-cadinol inhibited the mycelial growth of *F. oxysporum*, without majority compounds, whereas β-pinene has a smaller activity against this fungus. On other hand, Hazzit et al. (2009) consider that β-cadinol and γ-cadinene both are responsible for the activity against *Helicobacter pylori*, since these are the major compounds in essential oils of thyme. In the hexane extracts of *O. basilicum* (1.86%) and of *P.* Americana (0.9%) inhibition was at a low percentage, whereas β-cadinol, was found in the powders of *S. purpurea* (17.6%) and in the methanol extract of *O. basilicum* (1.9%). Although the percentage of β-cadinol in powders of *S. purpurea* was high, the effect on the development of *F. oxysporum* f. sp. *gladioli* was minor in comparison with the one of *P. americana* or *O. basilicum*, which indicates that the effect of this compoundis not related to the percentage in the extracts. Matasyoh et al. (2007) reported bactericidal and fungicidal activity of caryophyllene oxide, that was in the methanolic extracts of *P. americana* (3.15%), *P. guajava* (5.70%) and in the hexane extract of *S. purpurea* (2.26%). Most of these compounds belong to the family of the terpenes, which are abundant in essential oils of different species. These compounds appeared in lower concentrations in the different powders and extracts evaluated in the present work. Although, these compounds are not majority in the vegetal extracts, this does not exclude the fact that they are responsible for the observed activity, since as reported by Zheljazkov et al. (2008), the biological activity is not always dependent on the compounds present at the highest concentration, so the activity can be by some of compounds present at low concentrations or by a synergistic effect between them (Burt, 2004). The antifungal activity of the powders and extracts seems likely to be due to the synergistic effects of major and minor components of the powders and extracts.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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Eradication of biofilms formed by bacteria isolated from diabetic foot infections by potential antibiofilm agents alone and in combination with ciprofloxacin

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This study was performed to investigate the resistance of biofilm forming bacteria isolated from diabetic foot infection to different antibiotics and the eradicating activity of some potential antibiofilm agents alone and in combination with ciprofloxacin. Imipenem was the most active against biofilms formed by all tested strains, while tetracycline was the least active. For biofilms of Gram-positive bacteria, azithromycin and imipenem were the most potent, while tetracycline and vancomycin showed the lowest activity. Similarly, imipenem showed the highest activity against biofilms of Gram-negative bacteria, while ciprofloxacin, tetracycline and cefoperazone were the least active. Potential antibiofilm agents exerted antibacterial and biofilm eradicating activities. Apple and grape vinegars showed the highest activities, followed by estradiol, ambroxol and piroxicam. Dexamethasone, manuka and citrus honeys were less active. Ambroxol showed the highest synergistic activity with ciprofloxacin, followed by dexamethasone, manuka honey, piroxicam, estradiol and grape vinegar, while apple vinegar and citrus honey showed intermediate activity. In conclusion, this study recommends the use of antibiofilm agents in combination with antibiotics to combat the resistance of biofilms to antibiotics.

Key words: Diabetic foot infections, biofilm eradication, antibiofilm agents, ciprofloxacin, synergy.

INTRODUCTION

Diabetic foot infection (DFI) is a major problem in patients with diabetes. Reasons of this infection are peripheral neuropathy, reduced peripheral blood supply and lowered immunity. DFIs bear high risk for patients with diabetes because they may lead to gangrene and amputation (Abbott et al., 2002; CDC, 2005; Lauterbach et al., 2010). The microbial etiology of DFIs is complex. Resistance of bacteria causing DFIs to antibiotics is common and formation of biofilms complicates the problem (Roghmann et al., 2001). Biofilm is a community of sessile microbial cells attached to a surface and housed within a matrix of polysaccharides, proteins and nucleic acids (Hoiby et al., 2010).

Biofilms are remarkably resistant to antimicrobial agents. The mechanisms of biofilm resistance may include slow growth and metabolic rates, inactivation of

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Table 1. Bacterial strains used in this study.

Bacterial strains	Number
Proteus mirabilis	6
Proteus vulgaris	1
Pseudomonas aeruginosa	2
Pseudomonas mendocina	1
E. coli	3
Klebsiella ozaenae	1
Acinetobacter baumanii	1
Staphylococcus aureus	3
Staphylococcus epidermids	1
Entrococcus faecalis	1

antimicrobial agents by the extracellular matrix and the presence of an oxygen gradient that prevents the action of some antibiotics (Lynch and Robertson 2008; Hall-Stoodley and Stoodley, 2009). In addition, the biofilm matrix represents a diffusion barrier to antibiotics (Lynch and Robertson, 2008). Moreover, biofilms contain a large subpopulation of persister cells which are dormant cells that survive antimicrobial treatment (Lewis, 2010).

For these reasons, agents that can remove biofilms and act in synergism with antibiotics are urgently needed. This study investigated the *in vitro* activities of some potential antibiofilm agents alone and in combination with ciprofloxacin on the eradication of biofilms formed by bacterial isolates from diabetic foot infections.

MATERIALS AND METHODS

Media and chemicals

Tryptone soya broth, Tryptone soya agar and Mueller Hinton broth were the products of Oxoid (Hampshire, UK). Ambroxol hydrochloride, imipenem and Dimethyl sulphoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, USA). Antibiotics and chemicals used in this study were ciprofloxacin, amoxicillin-clavulinic acid and gentamicin (Egyptian Pharmaceutical Industries Company (EIPICO), 10th of Ramadan City, Egypt), Chloramphenicol (Alexandria Pharmaceutical and Chemical Industries Company, Alexandria, Egypt), tetracycline (Nile Pharmaceutical and Chemical Industries Company, Cairo, Egypt), cefoperazone, azithromycin and piroxicam (Pfizer, Cairo, Egypt), Manuka honey (Manuka health New Zealand Ltd., Te Awamutu, New Zealand), citrus honey (Isis Company, Egypt), estradiol and glutaraldehyde (El Nasr Pharmaceutical Chemicals Company, Cairo, Egypt), dexamethasone and cephalexin (Glaxo Smithkline, Cairo, Egypt), and vancomycin (Sigma Pharmaceutical Industries Company, Menoufia, Egypt). Apple and grape vinegar were purchased from the local market, Zagazig, Egypt. Other chemicals were of pharmaceutical grade.

Bacterial strains

Twenty isolates obtained from patients with diabetic foot infections admitted to the Surgery Department, Zagazig University Hospital were obtained from the stock culture collection of Department of Microbiology and Immunology, Faculty of Pharmacy, Zagazig University as shown in Table 1.

Quantitative assessment of biofilm by spectrophotometric method

The ability of tested strains to form biofilm was investigated according to Stepanovic et al. (2007). Overnight cultures of tested strains in Tryptone soya broth (TSB) were diluted with fresh TSB to a final inoculum of 1 × 10^6 CFU/ml. To the wells of 96-well sterile microtiter plates with rounded bottom, aliquots of 200 µl of the prepared suspensions were added and the plates were incubated for 24 h at 37°C. The contents of the microtiter plates were gently removed and the wells were washed 3 times with sterile phosphate buffered saline (PBS, pH 7.2). To fix adherent bacteria, aliquots of 200 µl of 99% methanol were added to the wells for 20 min. The wells were stained with 200 µl crystal violet (1%) for 20 min and the unbound dye was washed by distilled water. After air drying of the plates, the bound dye was eluted by aliquots of 160 µl of 95% ethanol. The optical densities of the stained adherent films were measured with a spectrofluorimeter (Biotek, USA) at 490 nm. Measurements were performed in triplicate and repeated 3 times. The cut-off optical density (ODc) was calculated as three times standard deviations above the mean OD of the negative control. The tested strains were classified according to the criteria of Stepanovic et al. (2007) into non-biofilm producer (OD ≤ ODc), weak biofilm producer (OD > ODc, but ≤ 2x ODc), moderate biofilm producer (OD>2x ODc, but ≤ 4x ODc), and strong biofilm producer (OD> 4x ODc).

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of tested antibiotics and potential antibiofilm agents was determined by the broth microdilution method according to Clinical Laboratory and Standards Institute Guidelines (CLSI, 2012).Standardized bacterial suspensions with a turbidity equivalent to that of 0.5 McFarland standard were prepared from overnight cultures in tryptone soya broth. The standardized bacterial suspensions were diluted to a cell density of 10^6 CFU/ml. Aliquots of 50 µl of the adjusted bacterial suspensions in Mueller-Hinton broth were added to the wells of a microtiter plate that contain aliquots of 50 µl of double the required dilutions of the tested agents in Mueller-Hinton broth. The plates were incubated at 37ºC for 20 h and the MIC was calculated as the lowest concentration of the tested agents that inhibited the visible growth in the wells.

Determination of minimum biofilm eradication concentration (MBEC)

The minimum biofilm eradication concentration was determined according to Ceri et al. (1999) with some modifications. Suspensions of the tested strains with a cell density of 1x10^8 CFU/ml were prepared in Tryptone soya broth (TSB) and diluted in TSB to a cell density of 5x10^6 CFU/ml. For biofilm formation, aliquots of 100 µl were inoculated into the wells of 96-well polystyrene microtiter plates and the plates were incubated for 24 h at 37ºC. The non-adherent cells were gently aspirated and the wells were washed three times with phosphate buffered saline (PBS). Aliquots of 100 µl of different dilutions of tested agents were transferred to the wells and the plates were again incubated for 24 h at 37ºC. The contents of the wells were removed and the wells were washed again. To resuspend the biofilms in the wells, aliquots of 100 µl of sterile phosphate buffered saline were added and the sides of the wells with a pipette tip were scrapped. To calculate

Table 2. Quantitative assessment of biofilm formation by bacterial isolates.

Isolate number	Optical density at 490 nm	Biofilm formation capacity
PM1	0.384	Strong
PM2	0.263	Strong
PM3	0.344	Strong
PM4	0.283	Strong
PM5	0.325	Strong
PM6	0.289	Strong
PV	0.289	Strong
PA1	0.281	Strong
PA2	0.346	Strong
K. ozaenae	0.308	Strong
SA1	0.333	Strong
SA2	0.351	Strong
SA3	0.338	Strong
SE	0.334	Strong
E. faecalis	0.316	Strong
AB	0.444	Strong
EC1	0.282	Strong
EC2	0.321	Strong
EC3	0.259	Strong
P. mendocina	0.346	Strong

PM, *Proteus mirabilis;* PV, *Proteus vulgaris*; PA, *Pseudomonas aeruginosa*; *K. ozaenae*, *Klebsiella ozaenae;* SA, *Staphylococcus aureus*; SE, *Staphylococcus epidermidis*; *E. faecalis, Enterococcus faecalis;* AB, *Acinetobacter baumanii*; EC, *E. coli*; *P. mendocina*, *Pseudomonas mendocina*

MBEC, 10 µl from each well was transferred onto Tryptone soya gar plates (TSA), incubated at 37°C for 24 h and MBECs were defined as the least concentrations that showed no growth on TSA.

Testing for synergy between potential antibiofilm agents and ciprofloxacin

For determination of the synergism of potential tested antibiofilm agents with ciprofloxacin, the same method of Ceri et al. (1999) was used, but instead of adding 100 µl of tested agent, aliquots of 50 µl of 1/2 MIC of antibiofilm agents were added to 50 µl aliquots of different dilutions of ciprofloxacin.

RESULTS

Assessment of biofilm formation

All tested strains were found to be strong biofilm forming (Table 2). The ODc was calculated as 0.064. According to the criteria of Stepanovic et al. (2007), the bacterial isolate is considered a strong biofilm-forming if the optical density is greater than 0.256.

Susceptibility of planktonic and biofilm cells to antimicrobial agents

Biofilm cells demonstrated higher resistance than planktonic cells to different antibiotics as demonstrated by the ratios of MBEC to MIC of antibiotics in Table 3. This ratio was lowest for imipenem (2-16) folds, followed by amoxicillin-clavulinic acid (2-32) folds, gentamicin (16-32), ciprofloxacin (8-64) folds, and was highest for tetracycline (4-256) folds. Considering biofilms formed by Gram-positive bacteria, highest resistance was found with vancomycin (1024) folds and tetracycline (32-256) folds, while low resistance was observed with azithromycin (4-8) folds and imipenem (4-16) folds, amoxicillin-clavulinic acid and cephalexin (8-32) folds each, gentamicin and ciprofloxacin (32 folds each), chloramphenicol (16-64) folds. Biofilm cells of Gram-negative bacteria were highly resistant to cefoperazone (4-512) folds and tetracycline (4-256) folds. Lower resistance was obtained with gentamicin (16-64) folds, ciprofloxacin (8-64) folds, chloramphenicol (16-32) folds, amoxicillin-clavulinic acid (2-32) folds, while imipenem showed the highest antibiofilm activity (2-8) folds.

Susceptibility of bacterial isolates to potential antibiofilm agents

Antibacterial and antibiofilm activities were found against planktonic bacteria (Table 4).Both apple and grape vinegars showed the highest activities, followed by

Table 3. Antimicrobial susceptibility of planktonic and biofilm cells.

Isolate number	Gentamicin			Ciprofloxacin			Chloramphenicol			Tetracycline			Amoxicillin/clavulinic acid		
	MIC	MBEC	MBEC/MIC	MIC	MBEC	MBEC/MIC	MIC	MBEC	MBEC/MIC	MIC	MBEC	MBEC/MIC	MIC	MBEC	MBEC/MIC
PM1	0.5	16	32	0.125	8	64	32	512	16	64	2048	32	1	32	32
PM2	1	32	32	0.25	16	64	64	1024	16	64	1024	16	64	128	2
PM3	16	512	32	2	128	64	64	1024	16	64	2048	32	32	256	8
PM4	16	512	32	2	128	64	64	2048	16	64	2048	32	512	2048	4
PM5	4	128	32	2	128	64	8	256	32	256	4096	16	8	64	8
PM6	1	32	32	1	128	64	2	32	16	1	256	256	1	32	32
PV	4	128	32	2	32	32	64	1024	16	16	2048	128	64	128	2
PA1	16	1024	32	2	128	64	256	4096	16	32	1024	32	1024	8192	8
PA2	8	256	32	32	256	8	256	4096	16	64	2048	32	1024	8192	8
K. ozaenae	32	1024	32	64	1024	16	256	4096	16	256	4096	16	32	256	8
SA1	0.25	8	32	1	32	32	8	256	32	1	256	256	0.5	16	32
SA2	0.5	16	32	1	32	32	4	128	32	2	512	256	0.5	16	32
SA3	0.5	16	32	1	32	32	8	256	32	2	512	256	0.5	16	32
SE	256	8192	32	1	32	32	64	1024	16	32	1024	32	0.5	16	32
E. faecalis	256	8192	32	1	32	32	4	256	64	32	1024	32	32	256	8
AB	256	8192	32	128	2048	16	128	2048	16	128	2048	16	1024	8192	8
EC1	16	1024	64	32	512	16	8	256	32	256	4096	16	32	256	8
EC2	1	32	32	1	32	32	2	32	16	256	4096	16	32	256	8
EC3	128	2048	16	128	4096	32	2	32	16	256	2048	8	128	1024	8
P. mendocina	1	32	32	1	32	32	4	128	32	64	256	4	16	128	8

PM, *Proteus mirabilis;* PV*, Proteus vulgaris*; PA, *Pseudomonas aeruginosa*; *K. ozaenae*, *Klebsiella ozaenae;* SA, *Staphylococcus aureus*; SE, *Staphylococcus epidermidis*; *E. faecalis, Enterococcus faecalis;* AB, *Acinetobacter baumanii*; EC, *E. coli*; *P. mendocina*, *Pseudomonas mendocina.*

oestradiol, ambroxol and piroxicam. Dexamethasone, manuka and citrus honeys were less active.

Synergy between ciprofloxacin and antibiofilm agents

Synergy was found between ciprofloxacin and different potential antibiofilm agents (Table 5). Ambroxol reduced MBEC of ciprofloxacin by 4-128 folds, grape vinegar by 2-64 folds, piroxicam by 2-32 folds, dexamethasone by 4-16 folds and apple vinegar and estradiol by 2-16 folds each.

DISCUSSION

In this study, the resistance of biofilm cells to antibiotics was higher than that of planktonic cells. The magnitude of biofilm resistance to individual antibiotics was measured by the ratio of MBEC/MIC expressed by ≥ 90% of the tested isolates. The resistance of biofilms formed by all tested strains was the least against imipenem (8 folds), followed by amoxicillin-clavulinic acid, gentamicin and chloramphenicol (32 folds each) and ciprofloxacin (64 folds). Resistance to tetracycline was the highest (256 folds) as shown in Figure 1. On the other hand the resistance of biofilms formed by Gram-positive strains was low against azithromycin (8 folds) and imipenem (16 folds) as shown in Figure 2. Intermediate resistance

Table 3. Contd.

Isolate number	Cefoperazone			Vancomycin			Imipenem			Azithromycin			Cephalexin		
	MIC	MBEC	MBEC/MIC	MIC	MBEC	MBEC/MIC	MIC	MBEC	MBEC/MIC	MIC	MBEC	MBEC/MIC	MIC	MBEC	MBEC/MIC
PM1	0.5	16	32	NT	NT	NT	2	8	4	NT	NT	NT	NT	NT	NT
PM2	256	1024	4	NT	NT	NT	4	8	2	NT	NT	NT	NT	NT	NT
PM3	64	1024	16	NT	NT	NT	2	8	4	NT	NT	NT	NT	NT	NT
PM4	32	1024	32	NT	NT	NT	4	8	2	NT	NT	NT	NT	NT	NT
PM5	16	32	2	NT	NT	NT	4	8	2	NT	NT	NT	NT	NT	NT
PM6	64	1024	16	NT	NT	NT	4	8	2	NT	NT	NT	NT	NT	NT
PV	2	1024	512	NT	NT	NT	2	8	4	NT	NT	NT	NT	NT	NT
PA1	8	512	64	NT	NT	NT	0.5	2	4	NT	NT	NT	NT	NT	NT
PA2	256	512	2	NT	NT	NT	64	256	4	NT	NT	NT	NT	NT	NT
K. ozaenae	256	4096	16	NT	NT	NT	1	8	8	NT	NT	NT	NT	NT	NT
SA1	NT	NT	NT	2	2048	1024	0.5	8	16	2	16	8	4	8	32
SA2	NT	NT	NT	1	1024	1024	1	8	8	2	16	8	32	512	16
SA3	NT	NT	NT	1	1024	1024	1	8	8	512	2048	4	4	128	32
SE	NT	NT	NT	1	1024	1024	2	8	4	256	1024	4	64	1024	16
E. faecalis	NT	NT	NT	0.5	512	1024	1	8	8	1024	8192	4	256	2048	8
AB	256	1024	4	NT	NT	NT	2	8	4	NT	NT	NT	NT	NT	NT
EC1	0.5	16	32	NT	NT	NT	0.5	2	4	NT	NT	NT	NT	NT	NT
EC2	0.5	16	32	NT	NT	NT	0.5	2	4	NT	NT	NT	NT	NT	NT
EC3	0.5	16	32	NT	NT	NT	0.5	2	4	NT	NT	NT	NT	NT	NT
P. mendocina	8	512	64	NT	NT	NT	0.5	2	4	NT	NT	NT	NT	NT	NT

Table 4. Antimicrobial and antibiofilm activities of potential antibiofilm agents.

Isolate number	Ambroxol (mg/ml)		Dexamethasone (mg/ml)		Piroxicam (mg/ml)		Estradiol (mg/ml)		Manuka honey (%)		Citrus honey (%)		Apple vinegar (%)		Grape vinegar (%)	
	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC
M1	0.47	0.94	4	2	1.25	0.625	0.25	0.25	25	9.375	12.5	18.75	0.078	0.31	0.078	0.156
M2	0.47	0.94	4	2	1.25	0.625	0.5	1	12.5	9.375	25	18.75	0.078	0.31	0.039	0.156
M3	0.47	0.94	4	2	1.25	0.625	0.5	1	12.5	9.375	12.5	18.75	0.078	0.31	0.039	0.156
M4	0.47	0.94	4	2	1.25	0.625	0.5	1	12.5	9.375	25	18.75	0.078	0.31	0.078	0.156
M5	0.47	0.94	4	2	1.25	1.25	0.25	0.5	12.5	9.375	12.5	18.75	0.078	0.31	0.078	0.156
M6	0.47	0.94	4	2	1.25	1.25	0.5	0.5	12.5	9.375	12.5	18.75	0.078	0.31	0.078	0.156
PV	0.47	0.94	4	2	0.625	1.25	0.5	1	12.5	9.375	12.5	18.75	0.078	0.31	0.039	0.156

Table 4. Contd.

PA1	0.47	0.94	4	2	1.25	0.625	0.25	0.25	12.5	9.375	12.5	18.75	0.078	0.156	0.039	0.156
PA2	0.47	0.47	4	2	1.25	0.625	0.25	0.5	12.5	9.375	12.5	18.75	0.625	0.31	0.039	0.156
K. .ozaenae	0.47	0.47	4	2	1.25	0.625	0.25	0.25	25	9.375	25	18.75	0.195	0.31	0.078	0.156
SA1	0.47	0.47	1	2	0.625	1.25	0.125	0.25	12.5	9.375	25	18.75	0.078	0.31	0.039	0.156
SA2	0.47	0.47	1	2	0.625	0.625	0.25	0.25	12.5	9.375	12.5	18.75	0.078	0.31	0.078	0.156
SA3	0.47	0.47	1	2	0.625	0.625	0.125	0.25	12.5	9.375	25	18.75	0.078	0.31	0.078	0.156
SE	0.94	0.47	1	2	0.625	0.625	0.125	0.25	25	9.375	25	18.75	0.078	0.156	0.078	0.156
E. faecalis	0.94	0.47	1	2	0.625	0.625	0.5	0.25	25	9.375	25	18.75	0.078	0.156	0.078	0.156
AB	0.94	0.47	4	2	0.625	1.25	0.125	0.25	12.5	9.375	25	37.5	0.078	0.156	0.039	0.31
EC1	0.94	0.94	4	2	1.25	1.25	0.5	1	12.5	9.375	25	18.75	0.078	0.31	0.078	0.156
EC2	0.94	0.94	4	2	1.25	1.25	0.5	1	12.5	9.375	25	18.75	0.078	0.156	0.078	0.156
EC3	0.47	0.47	4	2	0.625	0.625	0.25	1	12.5	9.375	25	18.75	0.078	0.156	0.078	0.156
P. mendocina	0.47	0.94	4	2	0.625	0.625	0.25	0.5	12.5	9.375	25	18.75	0.078	0.31	0.0195	0.156

PM, *Proteus mirabilis;* PV, *Proteus vulgaris*; PA, *Pseudomonas aeruginosa*; *K. ozaenae*, *Klebsiella ozaenae;* SA, *Staphylococcus aureus*; SE, *Staphylococcus epidermidis*; *E. faecalis, Enterococcus faecalis;* AB, *Acinetobacter baumanii*; EC, *E. coli*; *P. mendocina*, *Pseudomonas mendocina.*

Table 5. Effect of potential antibiofilm agents on biofilm eradication by ciprofloxacin.

Isolate number	MBEC (µg/ml)								
	CIP	Apple vinegar /CIP	Grape vinegar /CIP	Citrus honey /CIP	Manuka honey / CIP	Ambroxol / CIP	Piroxicam / CIP	Dexamethasone / CIP	Estradiol/CIP
PM1	64	32	32	32	4	8	16	4	32
P. *mendocina*	256	128	16	128	128	8	64	32	32
SA2	256	32	16	128	64	16	128	64	32
AB	2048	128	32	64	64	16	64	32	128
EC2	512	128	16	128	64	32	64	128	32
K. ozaenae	1024	128	32	128	64	16	64	64	64
SE	32	32	16	8	4	8	16	4	8
E. faecalis	64	32	16	32	4	8	16	8	32

PM, *Proteus mirabilis;K. ozaenae*, *Klebsiella ozaenae;* SA, *Staphylococcus aureus*; SE, *Staphylococcus epidermidis*; *E. faecalis, Enterococcus faecalis;* AB, *Acinetobacter baumanii*; EC, *E. coli*; *P. mendocina*, *Pseudomonas mendocina;* CIP, ciprofloxacin.

was observed against cephalexin, amoxicillin-clavulinic acid, ciprofloxacin and gentamicin (32 folds each), while it was high against tetracycline (256 folds) and vancomycin (1024 folds). Imipenem was the least affected by biofilms formed by Gram-negative bacteria (4 folds) as

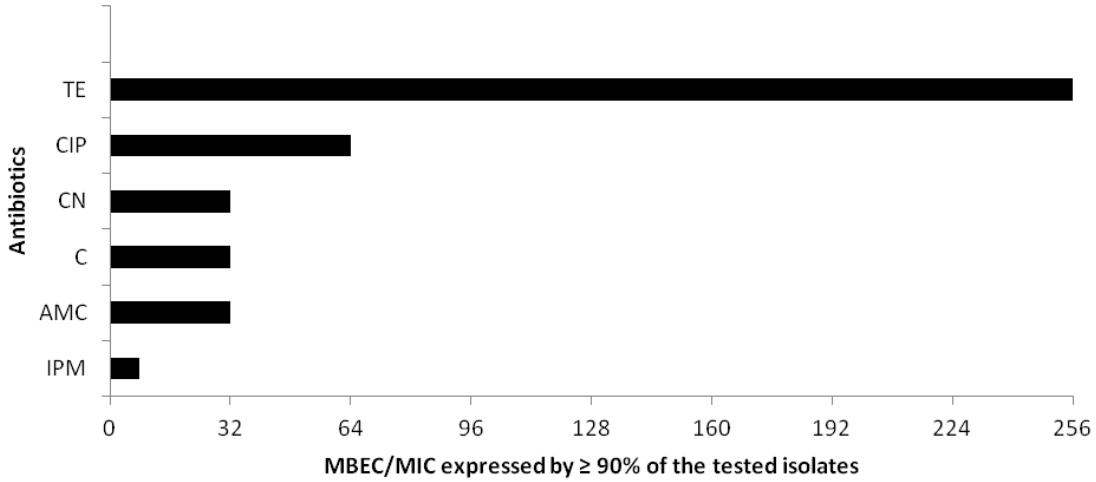

Figure 1. Biofilm eradicating activity of antibiotics against Gram-negative and Gram-positive bacteria IPM, imipenem; AMC, amoxicillin-clavulinic acid; C, chloramphenicol; CN, gentamicin; CIP, ciprofloxacin; TE, tetracycline

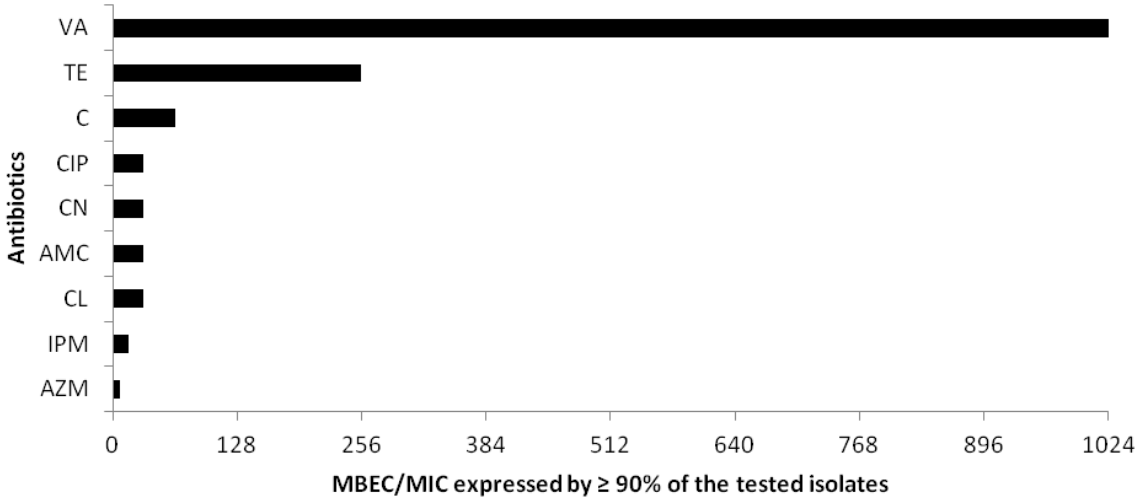

Figure 2. Biofilm eradicating activity of antibiotics against Gram-positive bacteria. AZM, azithromycin; IPM, imipenem; CL, cephalexin; AMC, amoxicillin-clavulinic acid; CN, gentamicin; CIP, ciprofloxacin; C, chloramphenicol; TE, tetracycline; VA, vancomycin.

shown in Figure 3. The biofilm resistance against chloramphenicol (16 folds), amoxicillin-clavulinic acid and gentamicin (32 folds each) was found to be intermediate, while the least active antibiotics against biofilm cells were ciprofloxacin and tetracycline (64 folds each) and cefoperazone (128 folds).

High resistance of biofilms to antimicrobial agents was reported by other studies. Thus Černohorská and Votava (2004) found that the susceptibility of biofilms formed by *E. coli*, *P. aeruginosa* and *Klebsiella pneumoniae* to cefoperazone and ciprofloxacin was much lower than that of planktonic cells. La Plante and Mermel (2009) reported that vancomycin was not effective for eradicating biofilms formed by *S. aureus* and *Enterococcus faecalis* as shown by MBEC/MIC ratios of ≥256 folds. Ceri et al. (1999) also reported high resistance of biofilms of *E. coli* to ciprofloxacin (MBEC/MIC >2048 folds), *P. aeruginosa* to ciprofloxacin (16 folds), gentamicin (64 folds) and imipenem (> 1024 folds), *S. aureus* to ciprofloxacin (1024 folds) and vancomycin (> 1024 folds).

As a result of the high resistance of biofilm cells to antibiotics, agents that can remove biofilms are necessary. A number of potential agents were tested. These agents include ambroxol, dexamethasone, piroxicam, manuka and citrus honeys, apple and grape vinegars and estradiol.

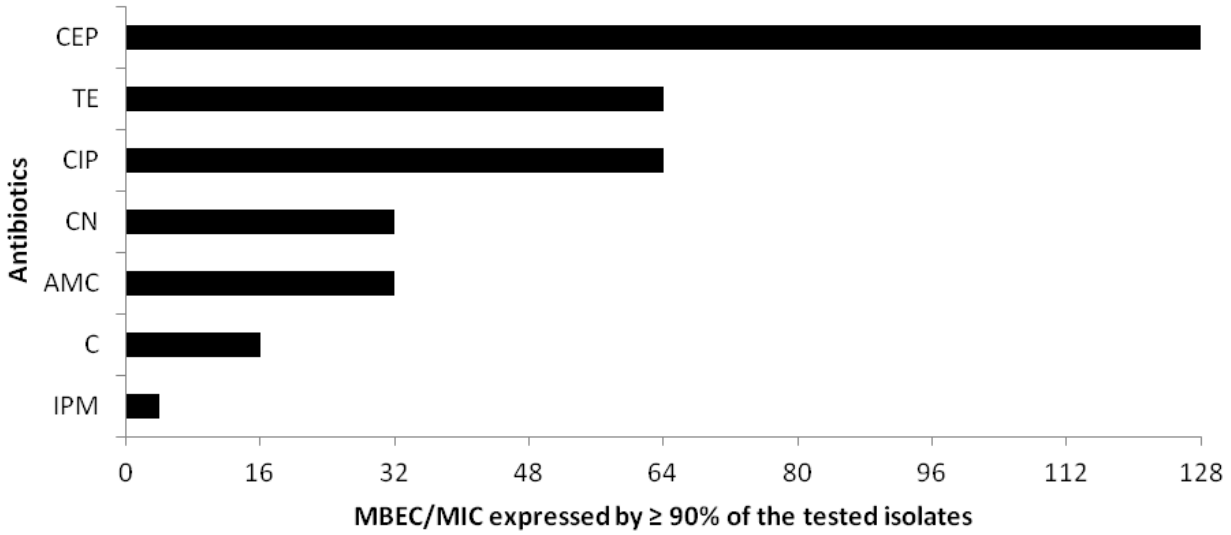

Figure 3. Biofilm eradicating activity of antibiotics against Gram-negative bacteria. IPM, imipenem; C, chloramphenicol; AMC, amoxicillin-clavulinic acid; CN, gentamicin; CIP, ciprofloxacin; TE, tetracycline; CEP, cefoperazone

Ambroxol was found to be a strong antiadhesion agent (Hafez et al., 2009). In addition to its antiahesive effects, ambroxol interferes with biofilm formation by interference with quorum sensing and decreasing the matrix production in *P. aeruginosa* biofilms (Li et al., 2008; Lu et al., 2010). Abbas (2013) also reported the ability of ambroxol to inhibit and eradicate biofilms formed by *Proteus mirabilis* isolated from diabetic foot infections. At 0.9 mg/ml, ambroxol caused 90.25-100% inhibition and 78.38-83.77% eradication of biofilm.

In this study, the MICs and MBECs of ambroxol against tested isolates were found to be 0.47-0.94 mg/ml. Lu et al. (2010) reported that at 1.875 and 3.75 mg/ml, ambroxol could inhibit quorum sensing, biofilm maturation and viability. Furthermore, Li et al. (2008) found that ambroxol at 3.75 mg/ml could disrupt the biofilms.

Honey has both a broad spectrum antibacterial and wound healing activities (Lusby et al., 2005). The antibacterial activity may be due to low water content, high osmolarity and low pH, hydrogen peroxide and non-peroxide phytochemical components of honey (Rhoads et al., 2008). Moreover, honey was reported to have antibiofilm activity (Saraf et al., 2009) that may be due to its quorum sensing inhibiting activity (Wang et al., 2012).

In this study, two types of honey were used; Manuka honey and citrus honey. Both showed comparable activity against planktonic growth, while Manuka honey was more active in biofilm eradication. Manuka honey is a broad spectrum antibacterial agent (Blair et al., 2009). In addition, it could detach established biofilms (Merckoll et al., 2009). On the other hand, citrus honey (20.3%) had a strong growth inhibiting activity against *S. aureus*, and intermediate activity against each of *P. aeruginosa*, *Klebsiella pneumoniae* and *E. coli* (Hegazy, 2011).

Vinegar is a sour liquid prepared by the fermentation of many fruits such as apples and grapes. Acetic acid is the main constituent of vinegar. Vinegar has bacteriostatic and bactericidal effect on microorganism (Entani et al., 1998; Nascimento et al., 2003). Its mechanism of action depends on penetration and disruption of the bacterial cell membrane (Parish et al., 2003; Yousef and Juneja 2003; Marriott and Gravani 2006). The high content of polyphenols contributes to the antimicrobial activity of apple and grape vinegars (Jafari et al., 2012). Vinegar could eradicate biofilm formed by *Candida albicans* on acrylic resin plates (Alberto et al., 2006). This may be due to polyphenols that were reported to inhibit streptococcal biofilm formation through inhibition of enzymes that produce exopolymers; a major component in biofilm (Sendamangalam, 2010).

In our study, grape vinegar produced slightly higher antibacterial and biofilm eradicating activities than apple vinegar. Apple vinegar could inhibit the planktonic growth at 0.078% except for *Klebsiella ozaenae* (0.195%) and one *P. aeruginosa* strain (0.625%), while grape vinegar produced similar effect at 0.039-0.078% except for *P. mendocina* (0.195%). Moreover, grape vinegar could eradicate biofilms of all tested strains at 0.156% except for *Acinetobacter baumanii* (0.31%), while apple vinegar MBECs ranged between 0.156-0.31%.

In this study, the non-steroidal anti-inflammatory agent piroxicam exerted slightly stronger antibacterial and antibiofilm effects than dexamethasone. The MICs of piroxicam and dexamethasone were 0.625-1.25 and 1-4 mg/ml, respectively. The biofilm eradication was achieved at 0.625-1.25 and 2 mg/ml for piroxicam and dexamethasone, respectively.

Non-steroidal anti-inflammatory drugs (NSAIDs) have

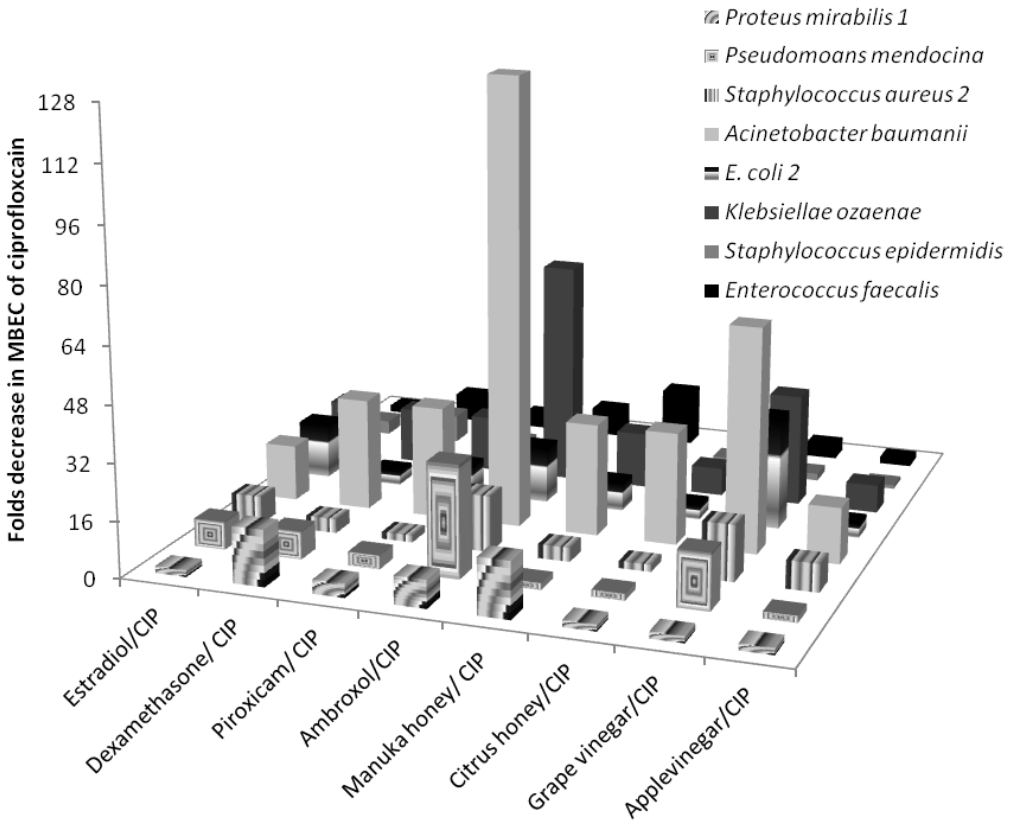

Figure 4. Effect of antibiofilm agents on biofilm eradication by ciprofloxacin.

good antimicrobial activities. This antimicrobial activity may be attributed to inhibition of bacterial DNA synthesis (Annadurai et al., 1998; Dastidar et al., 2000). Abbas et al. (2012a) found that piroxicam exerted antibiofilm activity against *P.aeruginosa* biofilms. Possible mechanisms of biofilm inhibition by NSAIDs are inhibition of bacterial adhesion, reduction of extracellular polysaccharide, modification of the surface properties of the bacterial cell (Farber and Wolff, 1992; Muller et al., 1998). Another possible mechanism is the inhibition of quorum sensing system. Piroxicam may inhibit biofilm formation by *P. aeruginosa* by decreasing the production of quorum sensing-dependent virulence factors (Ulusoy and Bosgelmez-Tinaz, 2013)

In our study, the steroidal hormone estradiol inhibited growth of free-living cells at 0.125-0.5 mg/ml and removed established biofilms at 0.25-1mg/ml. In a previous study, topical corticosteroids (fluticasone at 400 µg/200 µl, mometasone at 300 µg, 400 µg/200 µl and budesonide at 750 µg, 1,000 µg, and 2,000 µg/200 µl) were found to significantly reduce biofilms formed *in vitro* by *Staphylococcus aureus* isolated from chronic rhinosinusitis patients (Goggin et al., 2014).This activity may be due to the quorum sensing inhibiting activity of estradiol that was reported against *P. aeruginosa* (Beury-Cirou et al. 2013).

The synergistic effect of potential antibiofilm agents with ciprofloxacin was investigated. Ambroxol showed the highest synergistic activity (Figure 4). Ambroxol and dexamethasone showed synergistic effect against biofilms in all tested strains, but the magnitude of reduction in MBEC was higher in case of ambroxol. The synergistic effect was observed in 87.5% of tested strains with manuka honey, in 75% of isolates with each of piroxicam, estradiol and grape vinegar, but the magnitude of potentiation was greater with grape vinegar. Each of apple vinegar and citrus honey potentiated the biofilm eradicating activity of ciprofloxacin in 50% of isolates.

The biofilms formed by different strains were differently affected by antibiofilm agents-ciprofloxacin combinations. The most affected was *Acinetobacter baumanii* (all combinations showed synergism), followed by *Klebsiella ozaenae* and *E.coli* (all combinations showed synergism but with lower magnitude of MBEC decrease). Potentiation of the biofilm removal activity of ciprofloxacin was obtained by 6 combinations against *Staphylococcus aureus* biofilm, with 5 combinations against biofilms of each of *Pseudomonas mendocina*, *Enterococcus faecalis and Staphylococcus epidermidis*, but the magnitude of MBEC reduction was higher in *Pseudomonas mendocina* biofilm. The least affected was *Proteus mirabilis* biofilm; only 4 combinations showed synergism.

In accordance with our study, Li et al. (2008) found that ambroxol can increase the activity of ciprofloxacin against *P. aeruginosa* biofilms by increasing the permeability of ciprofloxacin. Abbas et al. (2012b) reported the potentiation of ciprofloxacin against established biofilms formed by 5 *P. aeruginosa* isolates by ambroxol. Synergistic activity of piroxicam with ciprofloxacin against pre-formed *P. aeruginosa* biofilms was also observed by Abbas et al. (2012c).

In summary, this study suggests that use of antibiofilm agents in combination with ciprofloxacin may be useful to overcome the high biofilm resistance to antibiotics, but further clinical trials should be done to test the clinical efficacy of such combinations.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Immobilization of dextranase by *Aspergillus penicillioides* NRC 39 and its properties

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Dextranase from *Aspergillus penicillioides* NRC 39 was immobilized using different carriers by various techniques including physical adsorption, covalent binding, ionic binding and entrapment. Immobilization of dextranase by covalent binding using 2% glutaraldehyde on prawn shell as carrier produced a high immobilization yield 87.4%. Comparison between the free and immobilized enzyme showed that immobilized enzyme on prawn shell produce highest immobilization yield at pH 6.0 and 40°C after 30 min of the reaction. Immobilized enzyme loses its activity when heated at 70°C for 40 min. The Km of free and immobilized enzyme was 15.8 and 17.4 mg/ml, respectively while V max of the free and immobilized enzyme was 28.5 and 23.8 U/mg protein, respectively.

Key words: Dextranase, immobilization, *Aspergillus penicillioides*.

INTRODUCTION

Dextran is a long chain carbohydrate polymer (1,6-glucosidic linkages), synthesized from glucose by the enzyme dextransucrase (EC 2.4.1.5). Various bacteria, fungi and other organisms are capable of producing dextran as an exopolysaccharide (Khalikova et al., 2005).

Dextranases [(1-6) X-D-glucan-6-glucanohydrolases; EC 3.2.1.11] are a group of hydrolytic enzymes that specifically hydrolyze the (1 - 6) linkages in dextrans (Abdel-Naby et al., 1999). Differences in molecular weight of dextran are of significant commercial interest in drug formulations, vaccines, cryoprotectants and stabilizers in the food industries, cosmetic products and as separating gels in research studies (Khalikova et al., 2005). Specific molecular weight fractions of dextran generated by dextranase are used to restore blood volume in patients suffering from shock as a result of severe blood loss (Mehvar, 2000).

Dextran is involved in dental plaque formation, so dextranases are used in the manufacture of dentifrices as an additive for presentation of dental carries (Kuboki et al., 1985) Dextranases also have other important industrial applications since these enzymes can depolymerise various troublesome microbial dextran deposits and reduce viscosity in sugar process. Dextran can be modified by dextranases to be used in many biotechnological applications.

Immobilized enzymes find broad application in industry, biotechnology, biomedicine and analytical chemistry (Yagiz et al., 2007 and Camacho et al., 2007). Generally, immobilized enzymes show better thermal and pH stabilities and are easier to separate, can be reused and their effect appears to be more suitable for practical applications (Ye et al., 2007).

Various techniques have been developed for enzyme immobilization, including adsorption to insoluble materials, entrapment in polymeric gels, encapsulation in membranes,

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cross linking with bifunctional reagent, or covalent linking to an insoluble carrier (Gomez et al., 2008; Hector et al., 2013).

Dextranase from *Penicillium funiculosum* 258 was immobilized on chitosan using glutaraldehyde for covalent binding. Comparison with the free and immobilized dextranase, the immobilized enzyme exhibited a higher pH optimum, optimal reaction temperature and thermal stability (Abdel-Naby et al., 1998).

Tanash et al. (2011) illustrated that *Aspergillus subolivaceus* dextranase could be immobilized on several carriers by entrapment and covalent binding with cross-linking. Dextranase immobilized on BSA with a cross-linking agent showed the highest activity .The optimum pH, temperature of the reaction and thermal stability were significantly improved by the immobilization process.

In the present study, *Aspergillus penicillioides* NRC 39 was immobilized on different carriers using different methods of immobilization including, physical adsorption, covalent binding, ionic binding and entrapment. The properties of both free and immobilized enzyme were compared.

MATERIALS AND METHODS

Microorganism

Aspergillus penicillioides NRC 39 was obtained from the culture collection of the Chemistry of Natural and Microbial Products Department at the National Research Centre, Dokki, Cairo, Egypt.

Culture medium and cultivation

The culture medium for enzyme production was prepared (Abdel-Nabyet al., 1998) as follows: (g/l) dextran with molecular weight (70000) (10.0), yeast extract (2.0), $NaNO_3$ (10.0), K_2HPO_4 (4.0), $MgSO_4$. 7 H_2 O(0.2), KCl (0.2) and $FeSO_4.7H_2O$ (0.01) (Pleszczynska et al., 1997).

Two discs (6 mm in diameter) from 7 days old cultures were transferred to 250 ml Erlenmeyer conical flasks each containing 50 ml fermentation medium. The inoculated flasks were incubated on a rotary incubator shaker at 180 rpm for 7 days at 28-30°C. At the end of incubation period, cultures were centrifuged at 8000 rpm. The cell free supernatant was used as a crude enzyme for further determinations.

Assay of dextranase activity

Dextranase activity was determined according to Miller (1959). The standard dextranase assay mixture contained 1 ml of 2% dextran in 0.1 M acetate buffer (pH 5.0) and 0.5 ml of suitably diluted enzyme solution. After 30 min incubation at 50°C the reducing sugars formed were analyzed by DNS method. One unit of enzyme activity was defined as the amount of enzyme that converts one micromole of isomaltose per minute reaction under the described condition.

Immobilization method

Physical adsorption

One gram of the carriers was inoculated 1 ml of enzyme from *A. penicillioides* NRC 39 (Abdel- Naby et al., 1998).

Covalent binding

One gram of the carriers was treated with 2 ml of 2.5% (v/v) glutaraldehyde for 2 h at 30°C. Washed with distilled water to remove the excess glutaraldehyde. The wet weight of carriers were mixed with 1 ml of enzyme solution and incubated overnight at 4°C. The unbounded enzyme was washed with distilled water (Abdel-Naby et al., 1998).

Ionic binding

One gram of cation was exchanged with acetate buffer (pH 5.0) or anion exchanger with phosphate buffer (pH 7.0). The carriers were incubated with 1 ml of enzyme solution at 4°C for 12 h (Abdel- Naby et al., 1998).

Entrapment immobilization

In agar and agarose

Ten milliliters of different concentrations of agar and agarose solutions (2.0, 2.5, 3.0 and 4.0 %) were mixed with 1.0 ml enzyme solution. The mixture was quickly solidified at 40°C, cut into small fragments and washed with 0.2 M acetate buffer (pH 5.0) to remove the unbounded enzyme (Wood Ward, 1985).

In calcium alginate

Ten milliliters of different concentration of calcium alginate (2.0, 2.5, 3.0 and 4.0%) were mixed with enzyme solution. One milliliter enzyme solution was added to 10 ml alginate, enzyme mixture was made into beads by dropping sodium alginate solution into 0.15 M of calcium chloride, the beads (0.5-1.0 mm diameter) were left for 2 h before collecting and washed with 0.1 M acetate buffer (pH 5.0), the unbounded enzyme was removed by washing with distilled water (Bicherstaff, 1997).

RESULTS AND DISCUSSION

Immobilization of dextranase obtained from *A. penicillioides* NRC 39

The culture filtrate from the optimized medium with the fungus was partially purified with ammonium sulphate which produced 1.1 fold of purification and specific activity, 8.2 U/mg protein while 70% acetone produced specific activity, 14.2 U/mg protein and 2.8 fold of purification.

Immobilization by physical adsorption

Immobilization of dextranase from *A. penicillioides* NRC 39 by physical adsorption was employed on different carriers including alumina, foam, chitin, loafacylinderica, prawn shell, pumice, sawdust, natural sponge and synthetic sponge. Results in Figure 1 show that prawn shell produced the highest immobilization yield (81.9%) with immobilization activity (113.3 U/g carrier) while alumina produced the lowest immobilization yield

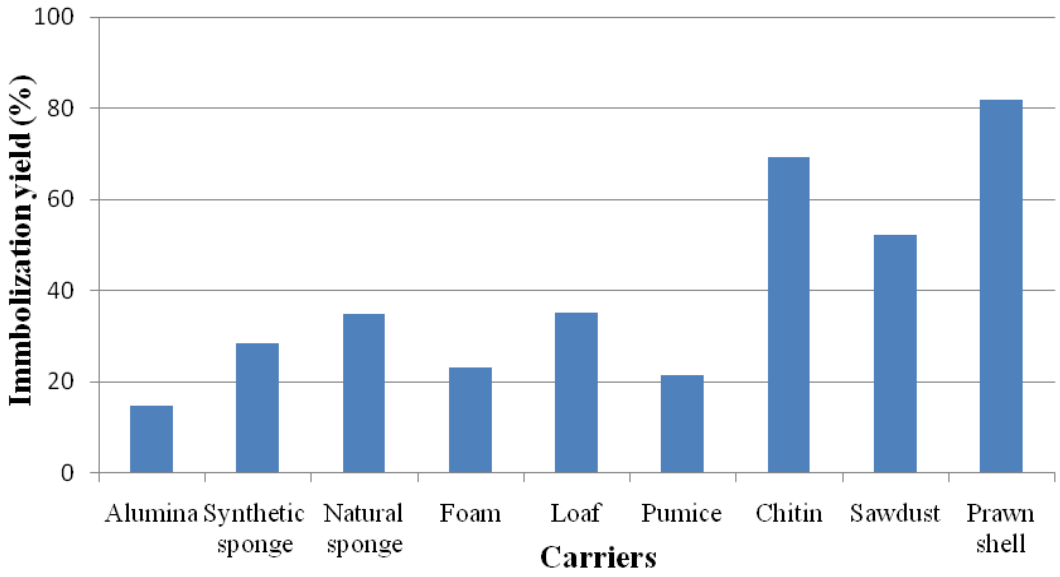

Figure 1. Immobilization of dextranase from *A. penicillioides* NRC 39 by physical adsorption.

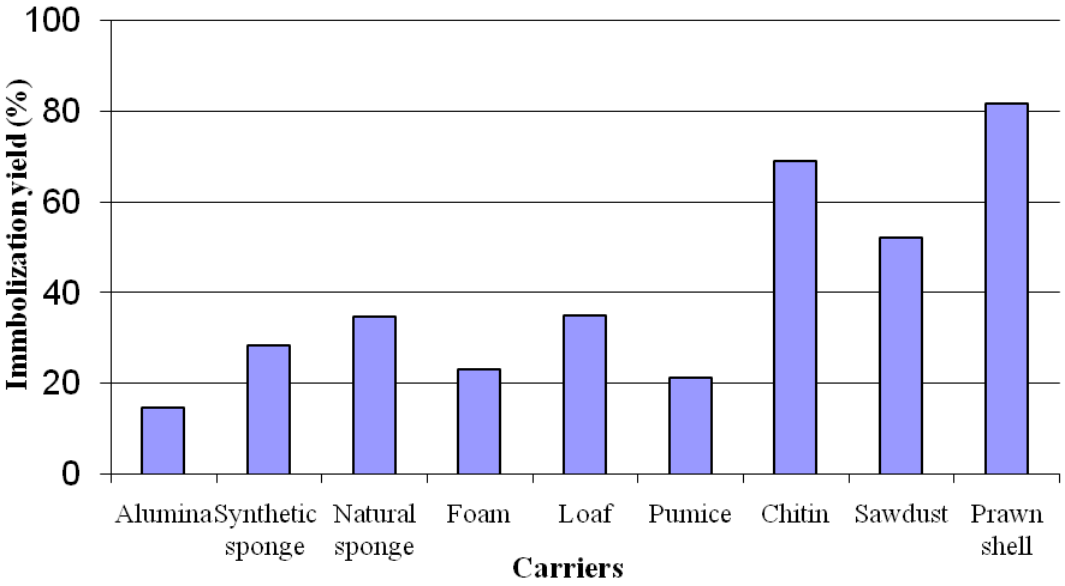

Figure 2. Immobilization of dextranase from *A. penicillioides* NRC 39 by covalent binding.

(14.7%), other carriers produced moderate to low immobilization yields. Abdel-Nabyet al. (1998) proved that the immobilized enzymes prepared by physical adsorption had the highest activity.

Immobilization by covalent binding

Results in Figure 2 showed that enzyme immobilization by covalent binding at prawn shell produce high immobilization yield (87.4%) with immobilization activity (124.2 U/g carrier). This high loading efficiency for the immobilization by covalent binding could be due to the formation of stable cross linking between the carrier and the enzyme through a spacer group which increased the local surface area of the carrier and reduced the steric hindrance around the active site of the enzyme molecule (Siso et al., 1990). These results were similar to those of Abdel-Naby et al. (1998) who reported that immobilization of *P. funiculosum* dextranase produced good immobilization

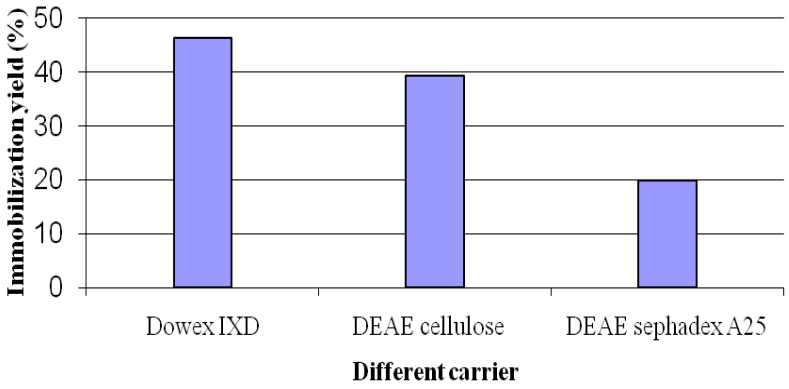

Figure 3. Immobilization of dextranase by ionic binding.

yield by covalent binding. Immobilizing enzymes by covalent binding result in an increase of the enzyme rigidity, which is commonly reflected by increase in the stability toward denaturation (Gottschalk and Jaenicke, 1991).

The presence of chitin, together with other polysaccharides, form fibrils of different lengths, depending on the species and the cellular location. In this work, a homopolymer was used with a broad spectrum of distribu-tion in the biosphere, being formed in the shells of crustaceans, such as crab, shrimp and lobster (Prasad et al., 2005).

Immobilization by ionic binding

The data illustrated in Figure 3 indicated that the highest enzyme yield (46.3%) was obtained using Dowex. On the other hand, the lowest immobilization yield (19.9%) was obtained by immobilizing the enzyme on sephadex, this inhibition may be due to the involvement of the fixation process to the active site of the enzyme (Galvez-Marisal and Lopez-Munguia, 1991).

Immobilization by entrapment

Immobilization of dextranase using different concentrations of agar, agarose and sodium alginate were examined. The results in Figure 4 show that 2% agarose produce the highest immobilization yield and immobilization decreased with increasing concentration of the carriers. These results agreed with those of Siso et al. (1990) who illustrated that decrease in yield with increase in carrier concentration might be due to the decrease in porosity of the gelmatrix which caused diffusion limitation for the substrate.

Properties of free and immobilized enzyme

pH of the reaction

The effect of different pH values of the reaction for free and immobilized enzyme on production of extracellular dextranase were investigated at the pH range of 4.0 to 7.0. Results in Table 1 show that the immobilized dextranase retain maximum activity at pH 6.0 while pH 5.0 was the optimal for the free enzyme. These results were the same as those of Galvez-Mariscal and Lopez-Munguia (1991) who found that the enzyme productivity of *Paecilomyces lilacinus* ranged from pH 5.4 to 7.0. Tanash et al. (2011) found that the optimum pH for activity of the immobilized enzyme of *A. subolivaceus* was shifted to pH 6.0 as compared to the free enzyme (pH 5.5). Shao-ying et al. (2013) found that the highest free dextranase activity was observed under the optimal reaction conditions of pH 5.5. These effects may be dependent on the ionic environment around the active site of the enzyme. Yakup and Aziz (2007) reported that the immobilization efficiency of dextranase was very high at pH 5.3.

Temperature of the reaction

Activity of free and immobilized dextranase was determined by incubation at different temperatures ranging from 20 to 80°C. Results in Table 2 show that the maximum dextranase activity was achieved at 40°C for immobilized enzymes, and at 50°C for free enzymes, increasing temperature above this range adversely affected the enzyme activity which lost 40% of activity at 80°C. The loss of enzyme activity might be due to low multiplication rate of the fungus biomass which ultimately decrease the enzyme production (Subasioglu and Cansunar, 2010).

Abdel Naby et al. (1999a) on the other hand reported that the optimal reaction temperature of dextranase from *Penicillium funiculosum* 258 was shifted from 60 for free to 80°C for the immobilized enzyme. Tanash et al. (2011) found that the optimal temperature of the reaction of dextranase from *A. subolivaceus* resulted at 60°C for both free and immobilized enzyme. This higher value of the optimal reaction temperature for the immobilized enzyme indicated that the applied immobilization procedure (covalent binding) contributed to greater

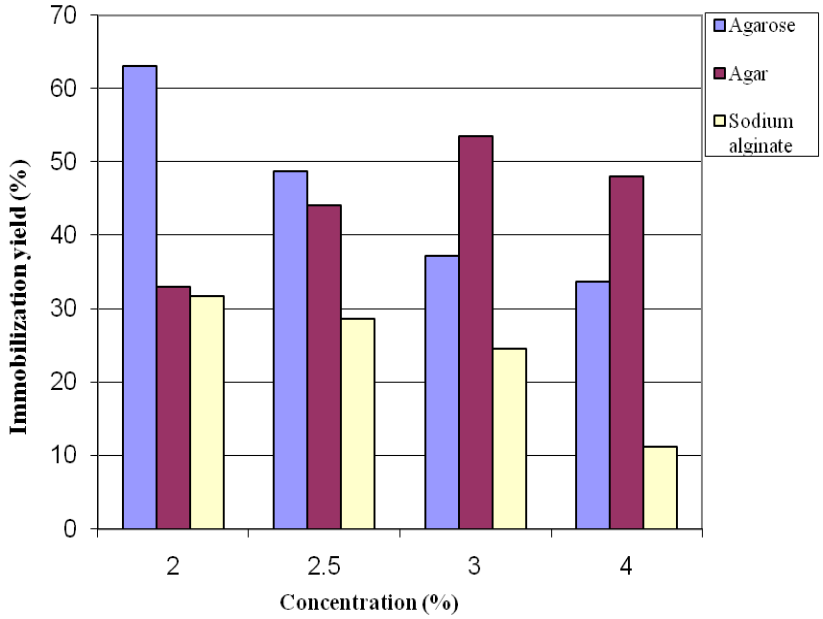

Figure 4. Immobilization of dextranase by entrapment.

Table 1. Effect of different pH values.

pH value	Relative activity (%)	
	Free enzyme	Immobilized enzyme
4	92.3	98.4
4.6	98.6	98.8
5.0 (control)	100	100
5.5	93.7	110.4
6	81.8	115.3
6.5	79	99.7
7	70	95.8

Table 2. Effect of different temperatures on the enzyme activity.

Temperature (°C)	Relative activity (%)	
	Free enzyme	Immobilized enzyme
20	34.1	87.2
30	63.9	103.2
40	84.3	112.1
50(control)	100	100
60	88.2	102.8
70	78.1	98.5
80	57.7	79.4

stability. Shao-ying et al. (2013) found that optimum temperature for free enzyme was 60°C.

Time of the reaction

The activities of the free and immobilized dextranase were assayed after incubation at various incubation times (15-90 min). As indicated in Figure 5 both the free and immobilized enzyme had maximum activity after 30 min. Increasing incubation time led to reduction in enzyme activity. Multiple fixation of the enzyme to matrix would also lead to a decrease in the activity due to the decrease in the catalytic activity (BicKerstaff, 1997).

Thermal stability

This experiment was designed to determine the effect of temperature on the activity of free and immobilized dextranases. Thermal stability of free and immobilized dextranases were investigated when incubated in the absence of substrate, at different temperatures ranging from 30 to 60°C, each for 10 to 60 min. The results illustrated in Figures 6 and 7 indicated that, immobilized enzymes was more stable than the free enzyme when heated at 30°C, the immobilization process protected the enzyme against heat inactivation. Immobilized enzyme lose its activity when heated at 70°C for 40 min. Whereas, the free enzyme was inactivated at a temperature of 60°C when heated for 40 min. Yakup and Aziz (2007) showed that *Penicillium lilacinum* dextranase thermal activity was enhanced by immobilization, soluble enzyme lost about 6 and 16% of its activity at 55 and 60°C, whereas immobilized enzyme retained 99 and 94% of its full activity at same temperature. In general, an immobilized enzyme is more stable than the free enzyme (Abdel-Naby et al., 1999a). This may be due to mass transfer resis-tance of the substrate into the carrier which particularly shows with a high molecular weight substrate like dextran.

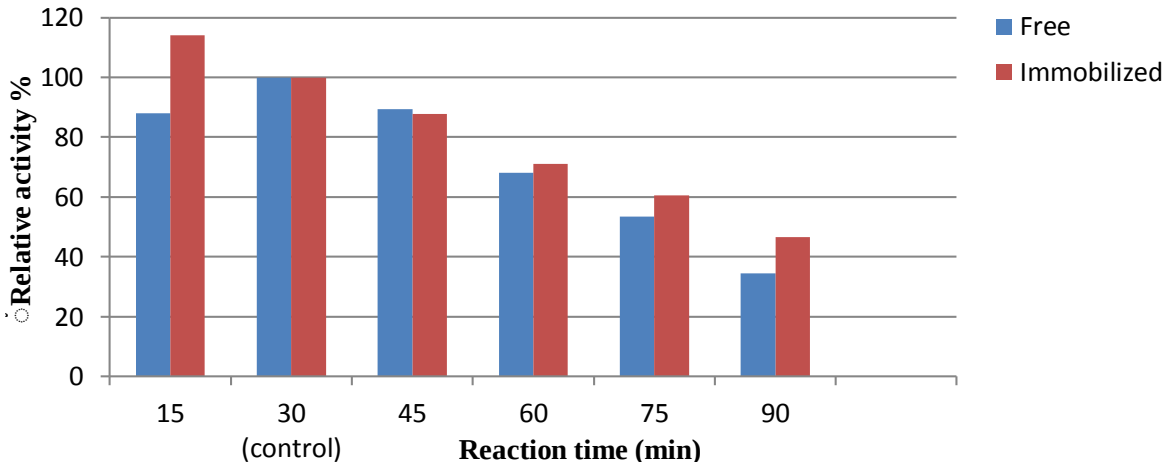

Figure 5. Effect of time of the reaction.

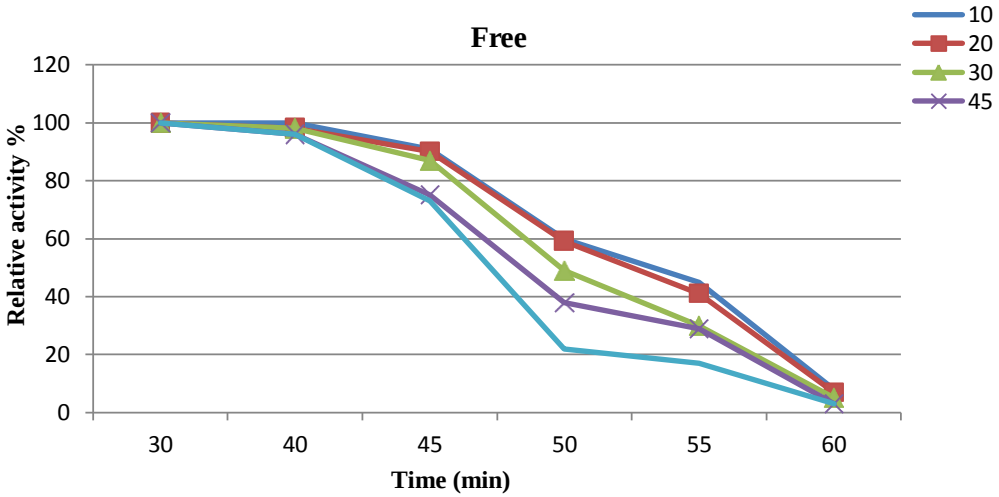

Figure 6. Thermal stability of free dextranase by *Aspergillus penicilliodies* NRC 39.

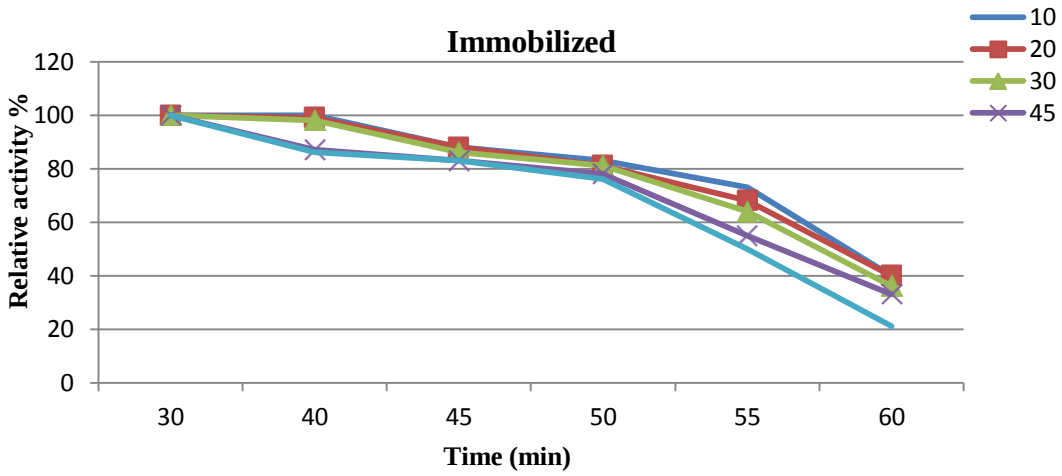

Figure 7. Thermal stability of immobilized dextranase by *Aspergillus penicillioides* NRC 39.

Table 3. Effect of metal salts on the activity of free and immobilized dextranase.

Different metal salts	Relative activity (%)	
	Free	Immobilized
Magnesium chloride	78.1	181.2
Zinc sulphate	85.3	80.2
EDTA	160.8	185.5
Cysteine Hcl	104.8	148.4
Copper sulphate	73.4	97.2
Cobalt chloride	97.2	90.6

Metal ions

Results in Table 3 indicated that relative activity of free enzyme increased in the presence of EDTA, while other metal ions decreased dextranase activity. On the other hand, EDTA, magnesium chloride and cysteine HCl increased relative activity of immobilized dextranase, other metal ions showed low activity. The results suggested that immobilization protected the enzyme against the inhibitory effects of some metal ions and inhibitors. These results are in agreement with those reported for other enzymes (Abdel-Naby et al., 1999a). The latter authors reported that the glycosylation of the enzyme formed a stable covalent bond that led to achievement of resistance against chemicals. The significant low activity of free enzyme may be attributed to direct contact between metal ions and the active site of enzyme. However, in immobilized enzyme, the fibrous porous structure play a role in protection due to the time required for these metal ions (Cu^{2+}, Hg^{2+} and Fe^{3+}) to diffuse to the carrier surface to reach the active site of enzymes (Abdel-Naby et al., 1999b). These results indicate the partial protection of the enzyme by immobilization is in agreement with those reported for other immobilized enzymes (Kimura et al., 1989).

Determination of Km and V max

Linweaver-Burk plots of the free and immobilized enzyme gave Km of 15.8 and 17.4 mg/ml, respectively. The V max of the free and immobilized enzyme were 28.5 and 23.8 U/mg protein, respectively.

The increase of the Km value after the immobilization may be due to mass transfer resistance of the substance into the immobilization matrix and to low substrate accessibility to the enzyme active site. Increasing the Km value of other enzymes after the immobilization has been reported by Abdel-Naby et al. (1999a). On the other hand, fixation of the enzyme on the immobilization matrix would lead to decrease in the flexibility of the enzyme molecule, which is commonly reflected by a decrease in the catalytic activity as reported by Erarslan et al. (1996). Consequently, the maximum reaction rate of the immobilized enzyme was lower than that of the free enzyme.

Conclusion

Prawn shell was a suitable carrier for immobilized dextranase from *A. penicillioides* NRC 39 by covalent binding using 2% glutaraldehyde resulting in the highest immobilization yield (87.4%). Immobilized enzyme exhibited a higher activity at optimum pH, temperature and time of reaction. Dextranase thermal activity was enhanced by immobilization. Protection of enzyme against inhibitory effects of some metal ions and inhibitors was indicated by immobilization. Km and V max of free and immobilized enzyme was determined.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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African Journal of Microbiology Research

Full Length Research Paper

In silico identification of 44 species and subspecies of staphylococci by restriction analysis of the *gap* gene polymorphism using *Hpy*CH4V enzyme

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The aim of this study was to detect and analyze *in silico* species/subspecies polymorphisms in the sequences of staphylococcal *gap* gene using *Hpy*CH4V restriction enzyme. We analyzed 64 deposited in the GenBank sequences of the *gap* gene, derived from 41 staphylococcal species and 23 staphylococcal subspecies. We also attempted to develop and describe specific RFLP profiles of the *gap* gene for each of the analyzed staphylococcal species and subspecies. Investigation by the present study revealed that *Hpy*CH4V restriction enzyme obtained 44 distinctive restriction profiles of the *gap* gene for 36 staphylococcal species, and moreover 8 subspecies represent further 4 species. This is the first report concerning the possibility to use a single restriction enzyme to distinguish and identify 44 species and subspecies of staphylococci.

Key words: *gap* gene, *Hpy*CH4V, *in silico*, RFLP, staphylococci.

INTRODUCTION

Currently, the genus *Staphylococcus* is divided into 48 species and 23 subspecies (Al Masalma et al., 2010; Hauschild et al., 2010; Nováková et al., 2010; Riesen and Perreten, 2010; Supré et al., 2010; Bergeron et al., 2011; De Bel et al., 2013; Pantůček et al., 2013). Both coagulase-positive and coagulase-negative staphylococci display wide inter- and also intra-species phenotypic diversity (Yugueros et al., 2000; Layer et al., 2007; Bergeron et al., 2011). Recently, numerous reports have implicated a wide range of staphylococcal species as etiological agents associated with infectious processes. The increasing clinical significance of staphylococci, make their accurate identification very important. However, despite increased recognition of the significance of infections attributed to staphylococci, many species cannot be identified using currently available methods.

Among the phenotypic methods for identification of staphylococci, there are biochemical profile analysis (Schwarzkopf et al., 1993; Lange et al., 1999) or phage typing (Schwarzkopf et al., 1993). Because of the variable expression of the phenotypic characters, these methods have frequently failed to make a reliable distinction between the different species of

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Staphylococcus (Martineau et al., 2001; Becker et al., 2004; Layer et al., 2007; Al Masalma et al., 2010; Bal et al., 2010). Instead or in addition to phenotypic methods, different test based on the bacterial genome analysis can be used. The advantage of genotypic methods over phenotypic tests was demonstrated in numerous studies (Martineau et al., 2001; Heikens et al., 2005; Layer et al., 2006). The most frequently genotypic methods used for identification of staphylococcal species are: PCR, RAPD-PCR (Vandenesch et al., 1995), PCR-RFLP (Yugueros et al., 2000; Layer et al., 2007), PCR amplicon-sequencing (Bergeron et al., 2011), ribotyping (Regnault et al., 1997) and DNA-DNA hybridization (Švec et al., 2004).

The *gap* gene has been widely considered a specific molecular marker for the *Staphylococcus* genus and analysis of the polymorphism of this gene as an effective method for the differentiation of closely related staphylococcal species (Yugueros et al., 2000, 2001; Layer et al., 2007; Ghebremedhin et al., 2008; Bergeron et al., 2011). The *gap* gene was firstly identified as a part of the glycolytic operon in *S. aureus*, however currently it is well known, that this gene also occurs in other staphylococcal species (Modun et al., 1994; Modun and Williams, 1999; Yugueros et al., 2000). The *gap* gene encodes a 42 kDa transferrin binding protein (Tpn) belonging to the family of multifunctional cell wall-associated glycerahdehyde-3-phosphate dehydrogenases, which catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate (Modun et al., 1994; Modun and Williams, 1999; Yugueros et al., 2000).

One of the most commonly used method to evaluate the polymorphism within the *gap* gene is PCR-RFLP. However, interpretation of the *gap* gene restriction patterns obtained for analyzed microorganisms requires comparison with the specific profiles derived from the reference strains or obtained on the basis of *in silico* analysis. Therefore, the aim of this study was to detect and analyze *in silico* species/subspecies polymorphisms of staphylococcal *gap* gene sequences available in GenBank using *Hpy*CH4V restriction enzyme.

Furthermore, we also attempted to develop and precisely describe characteristic restriction profiles of *gap* gene for each of the analyzed staphylococcal species/-subspecies. To our knowledge, this is the first report showing discriminatory potential of *Hpy*CH4V restriction enzyme in identification of staphylococcal species.

MATERIALS AND METHODS

Nucleotide sequences of the *gap* gene

The sequences of the *gap* gene were selected among all staphylococcal sequences published in GenBank database (accessed 25.04.2014). The selection of the sequences was based on the origin of strains for which the sequence was available (firstly sequences for reference strains were chosen) and the length of deposited sequences (longer sequences were preferred). In total, 64 sequences of the *gap* gene for 41 staphylococcal species and 23 subspecies were selected (Table 1).

Designing of primers and restriction analysis

In order to determine the most polymorphic regions in the sequences of *gap* gene of staphylococcal species and subspecies, the multiple sequence alignment using ClustalW software (Larkin et al., 2007) was performed. The primers with a length of 23 nucleotides (F: 5' ggtagaattggtcgtttagcatt 3', R: 5' gacatttcgttatcataccaagc 3') were designed for *S. aureus gap* gene sequence (GenBank, acc. no. AJ938182, 1011 bp) using Primer3 software.

The primers were aligned in the position of 31-53 nt and 934-956 nt of *S. aureus* AJ938182 *gap* gene sequence, and the length of amplified DNA product was 926 bp (Figure 1). Complementarity of the designed primers with the sequences of *gap* gene of other staphylococcal species or subspecies was analyzed using BioEdit software (Hall, 2007). In the case of sequences of staphylococcal species and subspecies shorter than 926 bp, the alignment with the sequence of the *gap* gene from *S. aureus* AJ938182 or counterparts species limited by primers was performed.

In silico restriction analysis was performed using NEBcutter V2.0 (Vincze et al., 2003) and HpyCH4V enzyme.

RESULTS

The lengths of the analyzed *gap* gene sequences together with designed primers for analyzed staphylococcal species or subspecies were 923, 925 or 926 bp (Table 2). Based on the comparative analysis (ClustalW), the differences in the lengths of these fragments resulted from the presence of mutation caused by insertion or deletion of one or three nucleotides.

In silico restriction analysis showed, that *Hpy*CH4V enzyme allowed to obtain 44 distinctive RFLP patterns of the *gap* gene for 36 species and for 8 subspecies of staphylococci belonging to another 4 species: *Staphylococcus capitis*, *Staphylococcus cohnii*, *Staphylococcus saprophyticus* and *Staphylococcus schleiferi* (Table 2 and Figure 2).

However, it was found that this restriction enzyme did not allow for the differentiation between *Staphylococcus jettensis* and *Staphylococcus petrasii* and also for intraspecies differentiation within 7 species: *Staphylococcus aureus*, *Staphylococcus carnosus*, *Staphylococcus equorum*, *Staphylococcus hominis*, *Staphylococcus petrasii*, *Staphylococcus sciuri* and *Staphylococcus succinus*. In this case, performed *in silico* restriction analysis of the *gap* gene sequences for 15 subspecies belonging to those species resulted in only species specific RFLP patterns (Table 2 and Figure 2).

DISCUSSION

Due to low discriminatory power, the phenotypic methods are often considered insufficiently reliable tool for the identification of staphylococci. For this reason, different genetic methods are increasingly used for their differentiation (Layer et al., 2006; Kim et al., 2008). Yugueros et al. (2000) first recommended the *gap* gene as a molecular marker for detection of *Staphylococcus* genus. The PCR primers sequences for amplification of this gene designed by those authors were also later used by e.g. Layer et al. (2007), Ghebremedhin et al. (2008), Bal et al. (2010) and Bergeron et al. (2011). However,

Table 1. The source and the length of analyzed *gap* gene sequences of *Staphylococcus* species and subspecies.

	Species / subspecies	GenBank accession number	Length of sequence (bp)		Species / subspecies	GenBank accession number	Length of sequence (bp)
1	*S. arlettae*	DQ321674	931	33	*S. jettensis*	JN092103	907
2	*S. aureus*	AJ938182	1011	34	*S. kloosii*	DQ321691	931
3	*S. aureus* subsp. *anaerobius*	HM352968	883	35	*S. lentus*	DQ321692	931
4	*S. aureus* subsp. *aureus*	CP000255	1011	36	*S. lugdunensis*	DQ321693	934
5	*S. auricularis*	DQ321675	931	37	*S. lutrae*	HM352978	880
6	*S. capitis*	DQ321676	934	38	*S. muscae*	DQ321694	931
7	*S. capitis* subsp. *capitis*	EU659902	814	39	*S. nepalensis*	EU659922	811
8	*S. capitis* subsp. *urealyticus*	HM352966	883	40	*S. pasteuri*	HM352972	880
9	*S. caprae*	DQ321677	934	41	*S. petrasii* subsp. *croceilyticus*	JX139896	880
10	*S. carnosus*	DQ321678	931	42	*S. petrasii* subsp. *petrasii*	JX139895	880
11	*S. carnosus* subsp. *carnosus*	EU659917	811	43	*S. pettenkoferi*	HM352976	880
12	*S. carnosus* subsp. *utilis*	EU659904	811	44	*S. piscifermentans*	AF495484	931
13	*S. chromogenes*	DQ321680	931	45	*S. pseudintermedius*	HM352982	880
14	*S. cohnii*	DQ321681	931	46	*S. saccharolyticus*	HM352969	883
15	*S. cohnii* subsp. *cohnii*	EU659921	811	47	*S. saprophyticus*	DQ321695	931
16	*S. cohnii* subsp. *urealyticus*	HM352971	880	48	*S. saprophyticus* subsp. *bovis*	HM352975	880
17	*S. condimenti*	EU659918	811	49	*S. saprophyticus* subsp. *saprophyticus*	EU659911	811
18	*S. delphini*	DQ321682	931	50	*S. schleiferi*	DQ321696	931
19	*S. devriesei*	JX174278	880	51	*S. schleiferi* subsp. *coagulans*	HM352980	880
20	*S. epidermidis*	DQ321683	934	52	*S. schleiferi* subsp. *schleiferi*	EU659913	811
21	*S. equorum*	DQ321684	931	53	*S. sciuri*	DQ321697	931
22	*S. equorum* subsp. *equorum*	EU659907	811	54	*S. sciuri* subsp. *carnaticus*	FJ578001	931
23	*S. equorum* subsp. *linens*	HM352977	880	55	*S. sciuri* subsp. *rodentium*	HM352984	880
24	*S. felis*	DQ321685	933	56	*S. sciuri* subsp. *sciuri*	FJ578000	931
25	*S. fleurettii*	EU659898	811	57	*S. simiae*	HM352970	883
26	*S. gallinarum*	DQ321686	931	58	*S. simulans*	DQ321698	931
27	*S. haemolyticus*	DQ321687	931	59	*S. succinus*	FJ578003	931
28	*S. hominis*	DQ321688	931	60	*S. succinus* subsp. *casei*	HM352981	880
29	*S. hominis* subsp. *hominis*	EU659908	811	61	*S. succinus* subsp. *succinus*	HM352974	880
30	*S. hominis* subsp. *novobiosepticus*	HM352973	880	62	*S. vitulinus*	EU659916	811
31	*S. hyicus*	DQ321689	931	63	*S. warneri*	DQ321699	931
32	*S. intermedius*	DQ321690	931	64	*S. xylosus*	DQ321700	931

due to the problems with amplification of the *gap* gene reported by several authors (Bal et al., 2010; Bergeron et al., 2011), in this work we decided to designed the new pair of primers on the basis of an *in silico* analysis of the64 sequences of gap gene available in the GenBank database

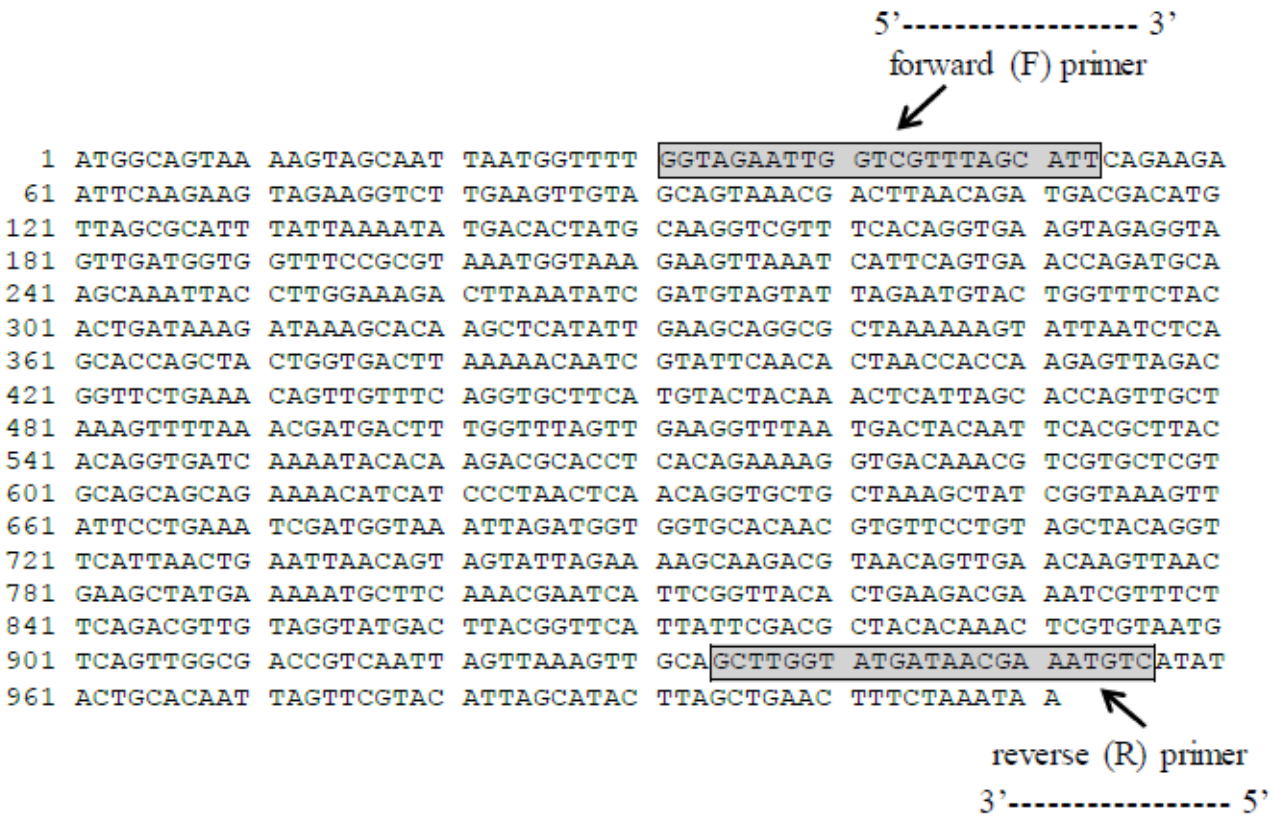

Figure 1. Primer annealing sites for the fragment of *S. aureus gap* gene sequence (GenBank Acc. No AJ938182).

Table 2. Restriction profiles of the *gap* gene of analyzed staphylococcal species and subspecies obtained after digestion with *Hpy*CH4V restriction enzyme (NEBcutter V2.0).

Species/subspecies	Molecular weight of the restriction fragments of the *gap* gene (bp)	Length of amplified product (bp)
S. arlettae	451, 180, 172, 120	923
S. aureus		
S. aureus subsp. *anaerobius*	363, 237, 120, 93, 88, 25	926
S. aureus subsp. *aureus*		
S. auricularis	211, 204, 135, 120, 99, 54, 39, 30, 25, 6	923
S. capitis	267, 219, 111, 96, 88, 61, 59, 25	926
S. capitis subsp. *capitis*		
S. capitis subsp. *urealyticus*	330, 144, 123, 120, 96, 88, 25	926
S. caprae	267, 186, 120, 96, 93, 88, 76	926
S. carnosus		
S. carnosus subsp. *carnosus*	267, 165, 120, 96, 88, 76, 75, 36	923
S. carnosus subsp. *utilis*		
S. chromogenes	234, 211, 174, 94, 84, 76, 26, 12, 6, 6	923
S. cohnii	304, 267, 111, 84, 81, 76	923
S. cohnii subsp. *cohnii*		
S. cohnii subsp. *urealyticus*	304, 261, 111, 84, 81, 76, 6	923
S. condimenti	267, 240, 120, 96, 88, 51, 36, 25	923
S. delphini	237, 234, 123, 117, 94, 93, 25	923
S. devriesei	184, 147, 144, 93, 87, 66, 61, 59, 57, 25	923
S. epidermidis	330, 184, 144, 117, 61, 59, 25, 6	926

Table 2. Contd.

S. equorum		
S. equorum subsp*. equorum*	331, 276, 234, 76, 6	923
S. equorum subsp*. linens*		
S. felis	225, 210, 118, 99, 70, 69, 57, 26, 24, 21, 6	925
S. fleurettii	304, 294, 111, 99, 51, 39, 25	923
S. gallinarum	267, 184, 180, 120, 96, 51, 25	923
S. haemolyticus	288, 150, 96, 88, 84, 61, 59, 39, 27, 25, 6	923
S. hominis		
S. hominis subsp*. hominis*	261, 240, 96, 88, 76, 61, 59, 36, 6	923
S. hominis subsp*. novobiosepticus*		
S. hyicus	445, 141, 94, 72, 63, 39, 26, 25, 12, 6	923
S. intermedius	240, 237, 234, 94, 93, 25	923
S. jettensis *	240, 147, 120, 96, 88, 87, 57, 36, 27, 25	923
S. kloosii	267, 216, 184, 120, 72, 39, 25	923
S. lentus	415, 291, 156, 36, 25	923
S. lugdunensis	273, 201, 184, 120, 66, 51, 25, 6	926
S. lutrae	331, 105, 84, 81, 76, 72, 63, 36, 33, 25, 11, 6	923
S. muscae	295, 153, 117, 94, 76, 72, 39, 33, 26, 12, 6	923
S. nepalensis	331, 234, 135, 111, 81, 25, 6	923
S. pasteuri	366, 165, 120, 88, 54, 51, 36, 25, 18	923
S. petrasii subsp*. croceilyticus* *	240, 147, 120, 96, 88, 87, 57, 36, 27, 25	923
S. petrasii subsp*. petrasii* *		
S. pettenkoferi	451, 276, 61, 59, 51, 25	923
S. piscifermentans	357, 216, 120, 111, 88, 25, 6	923
S. pseudintermedius	327, 240, 237, 94, 25	923
S. saccharolyticus	330, 144, 123, 96, 88, 61, 59, 25	926
S. saprophyticus	231, 184, 120, 111, 96, 76, 69, 36	923
S. saprophyticus subsp*. saprophyticus*		
S. saprophyticus subsp*. bovis*	204, 184, 120, 111, 96, 76, 69, 36, 27	923
S. schleiferi	295, 153, 94, 87, 72, 57, 51, 33, 26, 25, 18, 12	923
S. schleiferi subsp*. schleiferi*		
S. schleiferi subsp*. coagulans*	295, 153, 117, 94, 57, 51, 42, 33, 26, 25, 18, 12	923
S. sciuri	415, 177, 156, 99, 51, 25	923
S. sciuri subsp*. carnaticus*		
S. sciuri subsp*. rodentium*		
S. sciuri subsp*. sciuri*		
S. simiae	330, 156, 120, 111, 96, 88, 25	926
S. simulans	276, 234, 123, 120, 88, 51, 25, 6	923
S. succinus		
S. succinus subsp*. casei*	261, 184, 165, 120, 111, 76, 6	923
S. succinus subsp*. succinus*		
S. vitulinus	543, 304, 51, 25	923
S. warneri	207, 165, 159, 90, 88, 76, 61, 59, 18	923
S. xylosus	267, 184, 180, 120, 96, 76	923

*Species/subspecies which were not distinguished.

(accessed 25.04.2014). It should be noted, that amplification of the *gap* gene allows only for classification of bacteria to the genus *Staphylococcus*. However, as it was demonstrated by several authors, further analysis based on the polymorphism within this gene can be used as a useful tool for the differentiation of particular staphylococcal species (Yugueros et al., 2000, 2001; Layer et al., 2007; Ghebremedhin et al., 2008; Bergeron

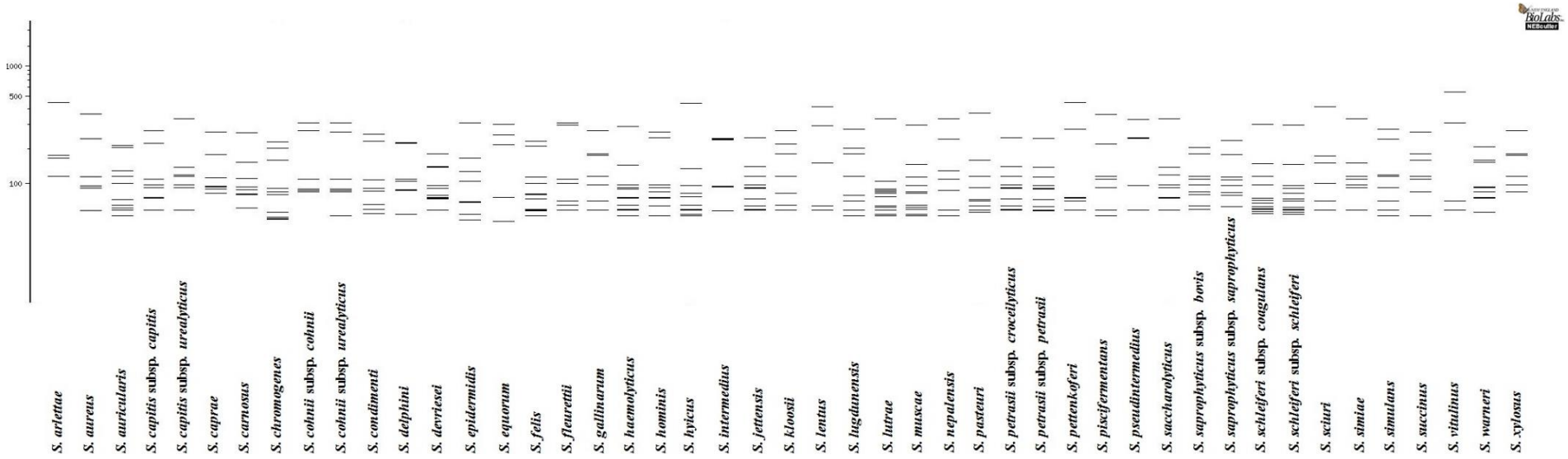

Figure 2. *In silico* generated RFLP patterns of the *gap* gene of analyzed staphylococcal species and subspecies obtained after digestion with *Hpy*CH4V restriction enzyme (NEBcutter V2.0).

et al., 2011).

The most widely studied and used restriction enzyme in the *gap* gene polymorphism analysis is *Alu*I. The PCR-RFLP of *gap* gene using *Alu*I for interspecies differentiation of staphylococci was firstly recommended by Yugueros et al. (2000). In their two studies, Yugueros et al. (2000, 2001), using *Alu*I enzyme were able to identify respectively 12 and 24 species of staphylococci. Expanding knowledge in this field, Bal et al. (2010) using *gap* gene compared discriminatory power of 3 endonuclease: *Alu*I, *Mse*I and *Rsa*I in the differentiation of coagulase-negative staphylococcal species. They showed that the *Alu*I enzyme has the highest discriminatory power to identify the 9 analyzed species of staphylococci. Furthermore, Layer et al. (2007) suggested terminal-restriction fragment length polymorphism of the *gap* gene using *Dde*I, *Bsp*HI and *Taq*I restriction enzymes, as a sensitive and reliable method that allows for the identification of 28 species of staphylococci.

Our *in silico* study demonstrate, that *Hpy*CH4V enzyme, as compared to other so far studied restriction enzymes used for the analysis of *gap* gene polymorphism is characterized by the very high discriminatory potential allowing for differentiation between 44 staphylococcal species and subspecies. According to thorough review of the available literature, the research concerning the application of *Hpy*CH4V restriction enzyme in detection of polymorphism of the *gap* gene and identification of staphylococcal species have not yet been performed. However it should be emphasized that our study was performed *in silico* and in the next step, in order to confirm the obtained results, the analogous laboratory analysis should be performed.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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