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α-L-ARABINOFURANOSIDASE FROM AN EFFICIENT HEMICELLULOLYTIC FUNGUS *Penicillium janthinellum* CAPABLE OF HYDROLYZING WHEAT AND RYE ARABINOXYLAN TO ARABINOSE

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ABSTRACT

This study reports *Penicillium janthinellum* strain, producing high levels of α-L-arabinofuranosidase (AFase) as well as other components of hemicelluloytic enzyme system (endoxylanase, β-xylosidase and acetyl xylan esterase) on rice straw and wheat bran containing solidified culture medium. Optimization of culture conditions was carried out using Box–Behnken design of experiment to study the influence of process variables (ammonium sulphate, pH and moisture level) on AFase production. Analysis of data showed R^2 (0.9967) and adjusted R^2 (0.9925) indicating model to be good fit and robust to predict culture conditions for AFase production. Under optimal culture conditions *P. janthinellum* produced high levels of AFase (212 units/g dw substrate) in addition to xylanase (1800 units/g dw substrate), β-xylosidase (31 units/g dw substrate), acetyl xylan esterase (231 units/g dw substrate) and feruloyl esterase (27 units/g dw substrate). AFase from *P. janthinellum* culture extract was purified to homogeneity and characterized to be a 64 kDa protein with a *pI* of 3.8. The peptide mass fingerprinting showed the AFase belonged to family GH54. The enzyme was optimally active at 50° C at pH 5.5 and its activity was positively modulated in presence of Fe^{3+}ions. The enzyme preferentially catalyzed the hydrolysis of *p*NP- arabinofuranoside (*p*NPA) with *Km* and *Vmax* of 0.4mM and 260 unit mg^{-1} $protein^{-1}$, respectively. Hydrolysis with purified AFase (3.0 units/g substrate) released arabinose from rye arabinoxylans (29.5 mg/g substrate) and wheat arabinoxylan (13.4 mg/g substrate), as the sole product indicating *P. janthinellum* as the important source of α-L-arabinofuranosidase for bioconversion of agro-residue to value added products.

Keywords: *Penicillium janthinellum;* hemicellulase; α-L-arabinofuranosidase; GH54; arabinoxylan hydrolysis; response surface methodology

INTRODUCTION

Arabinoxylans are integral hemicellulosic components of cereal crop residues and bioprocessing by-products from rice, wheat, rye, sorghum, barley, etc., **Lagaert *et al.* (2013)**. Arabinoxylans are heteropolymeric with xylan backbone comprised of β-1,4 linked xylose residues few of which are singly or doubly substituted with arabinosyl residues. While arabinosyl residues are further ester linked to ferulolyl moieties of lignin. Additionally xylose is also linked to acetyl residues at C2 position. Owing to its complex structure efficient hydrolysis of arabinoxylan requires collective action of an array of hemicellulolytic enzymes namely, endo-1,4 β-xylanase (EC 3.2.1.8) that cleaves the xylan backbone and releases xylo-oligomers, whereas, β-xylosidases (EC3.2.1.37) mediated catalysis results in release of monomeric xylose from xylo-oligomers. The α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.39), acetyl xylan esterase (EC 3.1.1.72) and feruloyl esterases (EC. 3.1.1.73) are involved in debranching of the heteropolymeric structure **(Shallom and Shoham, 2003; Dodd and Cann, 2009)**. The arabinoxylans derived from wheat and rye kernel are rich source of arabinose with xylose to arabinose ratio ranging between 0.49-0.5 **Guerfali *et al.* (2010)**. AFase plays an important role in hydrolysis of (1, 2), (1, 3) and (1, 5)-α-L-arabinofuranosyl moieties linked to xylan backbone of hemicellulose polymer and mitigate the steric hindrance for xylanase action **(Sorensen *et al.*, 2006)**. AFase derived from different microbial sources have been classified in GH families 3, 43, 51, 54, 62 and 93 (*Yang et al., 2015)*. AFase produced from *Aureobasidium pullulans*, *Trichoderma reesei* (**Numan and Bhosle, 2006**) and *Penicillium purpurogenum* (**Ravnal *et al.*, 2012**) have been reported as useful in bioconversion of lignocellulosic biomass to biofuel, paper and pulp industry and in wine industry for enhancement of wine flavor, respectively. This paper for the first time reports *P. janthinellum,* isolated from montane alpine forests of Shivalik hills (**Monga and Chadha, 2014**) India, as rich source of AFase and other components of hemicellulolytic enzyme complex. Culture conditions for achieving optimal AFase production were studied employing response surface methodology. The enzyme was further purified and characterized its role in efficient hydrolysis of arabinoxylans derived from wheat and rye for production of arabinose was established.

MATERIAL AND METHODS

Production of hemicellulases by *P. janthinellum*

P. janthinellum isolated from decomposing leaf litters of oak trees from montane alpine forests of Shivalik hills (India) was identified on the basis of morphological/ molecular approach (**Sharma *et al.*, 2008**). The fungus was grown at 30°C for 5 days on yeast potato soluble starch (YpSs) agar (Cooney and Emerson, 1964) and maintained on the same medium at 4°C. For production of hemicellulases *P. janthinellum* was grown on solidified culture medium containing rice straw and wheat bran (3:2) as carbon source and 15 ml basal medium (KH_2PO_4 0.4%, CH_3COONH_4 0.45%, and $(NH_4)_2SO_4$ 1.3%, pH 7.0). The flasks were inoculated with 2ml spore suspension (6×10^7 spores/ml) prepared from a 7 days old YpSs agar slants and incubated at 30 °C for 7 days. The enzyme was harvested by adding 50 ml of sodium citrate buffer (50 mM, pH 6.0) to the flasks and kept at 30 °C for 1 h under mild shaking. The resultant slurry was filtered and centrifuged at 8000×*g* for 20 min and the enzyme extract was used for assay of hemicellulolytic enzymes (xylanase, AFase, β-xylosidase and acetyl xylan esterase) as well as profiling AFase expression by SDS PAGE and 2 Dimensional gel electrophoresis, AFase activity staining of gels, and purification of enzyme.

The optimization of AFase was carried out using Box Benken Design of experiments employing response surface methodology. The production was studied using ammonium sulphate, pH and moisture level as independent variables at three levels (-1, 0, +1) using 17 flasks experiments. The experimental

data was analyzed using Statistical software (Design- Expert v 8.0.7, Stat-Ease Inc., USA) to calculate regression coefficient and generate response surface graphs.

Two dimensional gel electrophoresis (2DE)

The enzyme extract obtained after solidified culturing was subjected to ultrafiltration /desalting using 10 KDa membranes (Amicon). For 2DE protein sample (150 µg) was loaded onto IPG strips (7 cm) that rehydrated in buffer (150µl) containing 8 M urea, 2% CHAPS, destreak reagent, 1% IPG buffer (pH 3.0–10.0) and 0.005% bromophenol blue (BPB), for 16 h at room temperature. Ettan IGPhor 3 system (GE, Healthcare Biosciences) was used for IEF employing voltage program that increased linearly in a stepwise manner: 100 V, 4 h; 300 V, 2 h; 1,000 V, 2 h (gradient); 3,000 V, 3 h (gradient); 5,000, 3 h (gradient); 5000V, 6 h (step). Following IEF separation, IPG strips were equilibrated for 15 min in 7 ml of 0.05 M Tris Cl (pH 8.8), 8 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 60 mM dithiothreitol (DTT) and traces of bromo-phenol blue (BPB) followed by equilibration for 15 min in the same buffer where DTT was replaced with 50 mM iodo-acetamide. For second dimensional electrophoresis equilibrated IPG strips were placed onto 12% SDS, polyacrylamide gels and overlaid with 0.5 % low melting agarose solution that contains traces of BPB. The second dimension separation was carried using Hoefer mini VE system (GE Healthcare Biosciences) at a constant voltage of 25mA (**Kaur *et al*., 2013**). The protein spots were developed using silver staining method.

Identification of Protein

The well separated protein bands on SDS–PAGE were excised and subjected to peptide mass spectrometry analysis by 2D Nano LC/MS (Agilent 1100 series) at the TCGA (The Centre for Genomic Application, New Delhi). The data obtained by Mass spectrometry was compared with that present in Swiss Prot databases using the Mascot search algorithm.

Purification of AFase

The concentrated and desalted protein sample was loaded onto DEAE- Sepharose (fast flow) ion exchange column (24×2.6 cm; Pharmacia) pre equilibrated with sodium acetate buffer (50mM, pH 5.5). The column was first eluted with 2 bed volumes of equilibration buffer followed by a linear gradient of 1M NaCl in sodium acetate buffer (50mM, pH 5.5) at a flow rate of 1ml/min using (AKTA PRIME, Amersham Biosciences). Fractions corresponding to α-L-arabinofuranosidase active peak obtained during NaCl gradient elution were pooled, concentrated, desalted and equilibrated with 1.7M $(NH_4)_2SO_4$ in phosphate buffer (50mM pH 7.0) and applied onto phenyl sepharose (Amersham Biosciences) hydrophobic interaction (HIC) pre-packed column (5ml) that has been equilibrated with same buffer. The applied sample was eluted with a linear gradient of $(NH_4)_2SO_4$ (1.7M-0M) in phosphate buffer (50mM, pH 7.0) at a flow rate of 1ml/min. The fractions containing AFase activity were pooled, concentrated and desalted. AFase was further purified using sephacryl HR-200 (Amersham Biosciences) gel filtration (1.8× 90cm) column equilibrated with Tris-Hcl (20mM, pH 7.0) containing 0.15M NaCl. The protein in the fractions was determined by taking absorbance at 280 nm using Shimadzu-1240 spectrophotometer the fractions corresponding to purified AFase were pooled and characterized.

Assay of hemicellulolytic enzyme

Xylanase activity was assayed using 1% birchwood xylan (sigma) prepared in sodium citrate buffer (50mM, pH 6.0) as substrate. The assay was performed by incubating reaction mixture (1 ml) that contained equal amounts of appropriately diluted enzymes (500 µl) and substrate solution (500 µl) at 50 °C for 5 min. The reaction was terminated by adding 3ml DNS reagent and boiling for 10 minutes and developed colour was read at 540 nm. The reducing sugars were quantified using xylose as standard. One unit of xylanase activity was expressed as the amount of enzyme required to release 1 µmole of xylose per min under the assay conditions. The substrates, pNP-β-D-xylopyranoside, pNP-α-L-arabinofuranoside, (3 mM) prepared in sodium acetate buffer (50 mM, pH 5.0) were used to assay β-xylosidase, α-L-arabinofuranosidase (AFase), respectively using microtitre plate based method (Sharma *et al*., 2011). A reaction mixture (100µl) containing 50µl of sodium acetate buffer (50mM, pH 5.0), 25µl of suitably diluted enzyme, 25 µl of substrate (3mM) was incubated at 50° C for 30 min. The reaction was terminated by adding 100µl of NaOH-glycine buffer (0.4M, pH 10.8) and developed color was read at 405 nm using an ELISA plate reader (BioRad). The Afase and β-xylosidase activity was expressed in units as the amount of enzyme required to release 1 µmole of pNP per minute under assay conditions.

Characterization of AFase

SDS PAGE and Isoelectric focusing (IEF)

The homogeneity and molecular mass of AFase was determined by SDS-PAGE (12% gel) using Mini-Protean II system (BIORAD). Isoelectric focusing (IEF) was performed using a 5% acrylamide gel that contained 2.4% broad range pH range (2-10) ampholine carrier servalyte (SERVA, Germany). L-lysine (0.29%) and L-arginine (0.35%) were used as cathode buffers whereas ortho-phosphoric acid (10 mM) was used as anode buffer. IEF was carried out at constant voltage of 100V (1h) and 200 V (1h) and 500V for 30 min (**Badhan *et al*., 2004**).

Detection of AFase activity in gel using 4-methylumbelliferyl α-L-arabinofuranoside (MUA)

AFase activitiy in the gel was detected using 10 mM MUA as substrate (prepared in 50 mM sodium citrate buffer pH 6.0). Upon completion of electrophoresis, the gels were incubated in renaturation buffer [(20mM piperazine–N,N-bis (2-ethanesulfonic acid), 2mM dithiothreitol (DTT), 2.5mM $CaCl_2$, 2.5% Triton X-100] for 1h at room temperature and then overnight at 4° C in fresh renaturation buffer. After incubation the gel was thoroughly washed with sterilized double distilled water and incubated with overlaid substrate solution for 30 min at 50° C. The AFase activity spots were observed under UV light using gel documentation system (Gene Genius, Cambridge, UK).

Temperature and pH optima

The activity profile of the purified AFase was determined at a temperature between 30 and 90 ℃ with pNPA as substrate prepared in sodium acetate buffer (0.05 M, pH 5.0). The optimal pH was determined by measuring AFase activity between pH 2 and 10 using 0.1 M HCl-KCl (pH 2.0), sodium citrate (pH 3.0 to 6.0), sodium phosphate (pH 7.0 and 8.0), and Glycine-NaOH (pH 9.0 and 10.0 buffers at 50℃.

Thermal and pH stability of AFase

The aliquots of purified AFase were incubated at 50 ˚C and 60 ˚C at pH 5.0 and 6.0 for 0 - 4 h, and subsequently assayed for residual enzyme activity.

Effect of the metal ions and other reagents

The purified AFase was incubated in 5 mM solutions of NaCl, KCl, $MnCl_2$, $MgCl_2$, $CuCl_2$, $ZnCl_2$, $FeCl_3$, $CaCl_2$, EDTA, DTT, β-mercaptoethanol, NBS and SDS for 30 min at room temperature in sodium acetate buffer (0.05 M, pH 5.0). The residual AFase activity in the aliquots was assayed thereafter.

Substrate specificity

Substrate specificity of purified AFase was determined against 3mM *p*-nitrophenyl substrates, pNp-α-L-arabinofuranoside, pNp-α-L-arabinopyranoside, oNP-β-D-xylopyranoside, pNP-β-D-xylopyranoside pNP-β-D-glucopyranoside, oNP-β-D-galactopyranoside, pNP-β-D-glucoside, and pNP-cellobioside, pNP-α-D-mannopyranoside, 4-nitrophenyl-2-o-feruoryl-α-L-arabinofuranoside, 4-nitrophenyl-5-*O*-feruoryl-α-L-arabinofuranoside. (A reaction mixture (100µl) containing 25µl of substrate, 25µl of purified enzyme and 50µl of sodium acetate buffer (50mM, pH5.0) was incubated at 50° C for 30 min). The K_m, V_{max} and K_{cat} of purified AFase was determined against pNPA as substrate using Lineweaver Burke plot.

Analysis of Hydrolysis by Thin Layer (TLC) and High pressure liquid chromatography (HPLC)

900 µl of 1% w/v rye arabinoxylan (RAX), wheat arabinoxylan (WAX) and debranched arabinan (DA) prepared in sodium citrate buffer (50mM, pH 6.0) were incubated with 100µl of purified α-L-arabinofuranosidase at 50 ° C for 72 h. Samples were withdrawn at interval of 24 h, freeze dried and redissolved in methanol. Thin layer chromatography (0.25-mm layers of silica gel F-254 plates, Merck, India) was carried out using ethyl acetate: acetic acid: water as solvent system in a ratio of (3:2:1 v/v). The TLC plates were then sprayed with diphenylamine reagent and air dried. Hydrolysis products were visualized by heating plates at 100° C for 1-2 minutes. (1% w/v) Arabinose and xylose were run as standards. HPLC was carried out with DIONEX system (USA) using differential refractive index detector (RI-101, Shodex). The temperature of the Aminex column HPX-87P column (Bio-Rad) was maintained at 85° C and water was used as mobile phase at a flow rate of 0.6ml/min. (1% w/v) arabinose, and xylose were used as standards.

RESULTS

Production of Hemicellulolytic enzymes by *Penicillium janthinellum*

The results (Fig 1) show hemicellulolytic production profile of *P. janthinellum* grown on wheat bran/rice straw containing solidified culture medium. Culture produced maximal levels of xylanase (1079 units/gds), AFase (140 units/gds), β-xylosidase (15.4 units/gds) and AXE (136 units/gds) after 7 days of incubation. SDS-PAGE and corresponding activity profiling of AFase by developing zymogram also indicated to gradual increase in AFase expression as observed from intensity of bands (Fig 2a). AFase active protein band was observed corresponding to 65 KDa (Fig 2b). The resolution of secretome by 2-dimensional gel electrophoresis (pI 3.0-10.0) revealed well separated protein spots (Fig. 2c) and zymogram developed using MUA as substrate, showed three distinct AFase active spots of high molecular weight and acidic pI (Fig 2d). The spots were identified using Peptide mass fingerprinting using LC-MS/MS approach in which two spots were identified as AFase belonging to GH 54 family showing close similarity to AFase from *A. niger*($E=1e^{-09}$). The peptides matching were ADKWAIRGG, NSASVSLSTY.

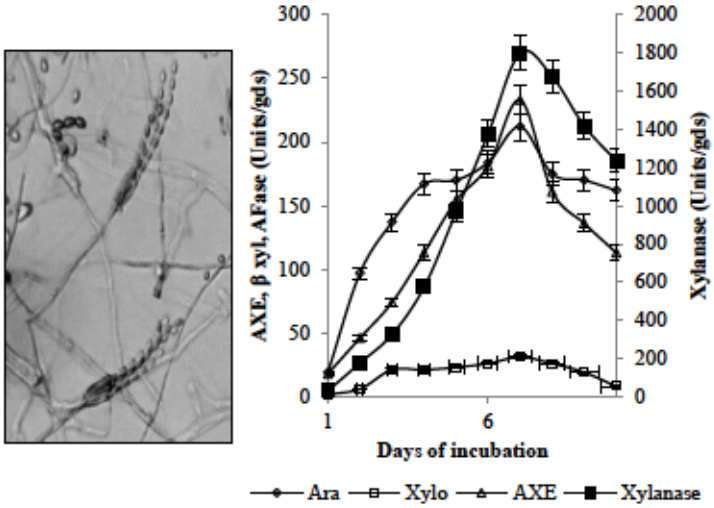


Figure 1 Production profile of hemicellulolytic enzymes (Arabinofuranosidase (AFase), β-xylosidase (β-xyl), Acetylxylan esterase (AXE) and Xylanase) produced by *Penicillium janthinellum* under unoptimized conditions. Error bars indicate SE @ 5% level.

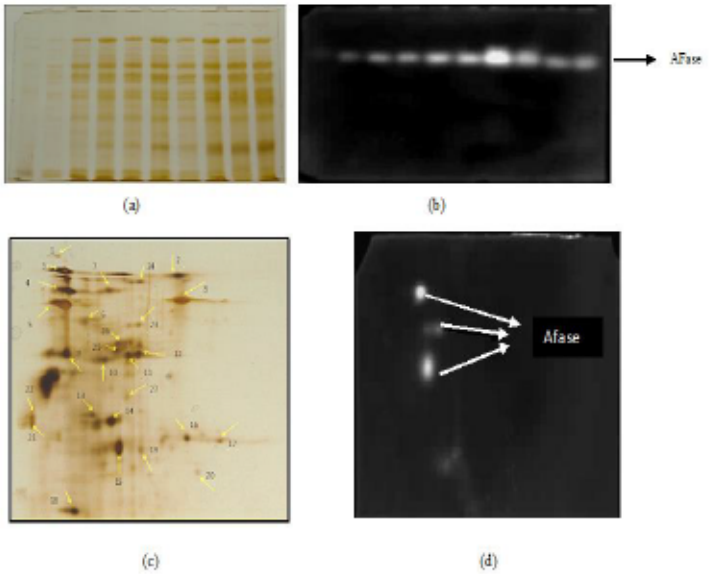


Figure 2 a) SDS-PAGE showing protein profile produced by *P. janthellium* under solid state fermentation under solid state fermentation at different day interval. b) Zymogram showing multiplicity of arabinofuranosidase developed by renaturation of SDS-PAGE. c) 2-DE secretome pattern of *P. janthinellum* grown on rice straw: wheat bran (3:2) containing solidified culture medium. Spots 3, 4 and 5 detected in 2D gel represents AFase activity d) Localization of α-L-arabinofuranosidase activity in 2DE gel.

Optimization of AFase production

The BBD experimental data showing effect of pH, moisture level and $(NH_4)_2SO_4$ on AFase production along with mean predicted and observed responses is presented in (Table 1). The model computed based on the data showed R^2 (0.997) and adjusted R^2 (0.992) along with high model F value of 236.43 and non significant lack of fit indicated it to be good fit to explain influence of effect of these variable on AFase production (Table 2). The production of AFase was significantly affected by $(NH_4)_2SO_4$, pH and moisture level in linear terms and squared terms ($p<0.005$). The iso-response contour plots (Fig. 3a, 3b) showed that maximal production of AFase (215 units/gds) could be achieved when the initial pH was in the range of 5.6-6.0, $(NH_4)_2SO_4$ concentration between 2.25-2.75 and moisture level was between 67-72%. In order to determine the accuracy of the model and to verify the optimization results, experiments were repeated three times under optimized culture conditions and AFase production of 211±3.2 (units/gds) was achieved that was 1.51 folds higher when compared to that achieved under unoptimized conditions. In addition to AFase, under optimized conditions production of other hemicellulolytic components (xylanase 1800 units/gds, β-xylosidase 31 units/gds, XAE 231 units/gds) (Fig 4) were also improved corresponding to 1.66, 2.01 and 1.73 fold increase, respectively. Table 3 shows the hemicellulolytic activities of different fungal strains under SmF and SSF. The enzyme activities obtained by *P. janthinellum* were comparatively higher to *Penicillium janczewskii*, *P. oxalicum*, *Aspergillus wortmanni*, *Talaromyces thermophilus*, *T. wortmanni* (under SmF) and *P. brasilianum and A. niger* NRRL 328 (under SSF).

Table 1 Box-Behnken design along with actual and predicted values of α-L-arabinofuranosidase.

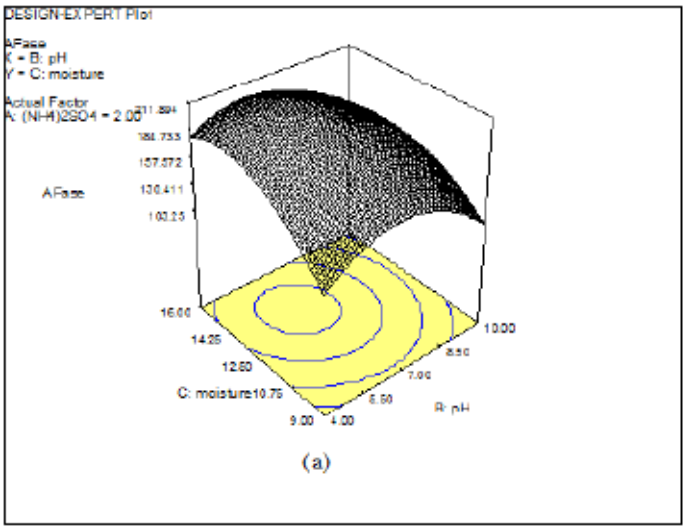
	Independent variables						Response 1	
	A	B	C	A:(NH4)2SO4	B:pH	C:Moisture level	Arabinofuranosidase	
Std				%		%	U/g ds	
	Coded levels			Actual level			Actual	Predicted
1	-1	-1	0	0.5	4	67	48.20	48.04
2	1	-1	0	3.5	4	67	170.10	175.59
3	-1	1	0	0.5	10	67	70.10	64.61
4	1	1	0	3.5	10	67	89.00	89.16
5	-1	0	-1	0.5	7	59	42.40	43.64
6	1	0	-1	3.5	7	59	121.60	117.19
7	-1	0	1	0.5	7	75	77.80	82.21
8	1	0	1	3.5	7	75	162.00	160.46
9	0	-1	-1	2.0	4	59	119.00	117.90
10	0	1	-1	2.0	10	59	99.00	103.25
11	0	-1	1	2.0	4	75	183.50	179.25
12	0	1	1	2.0	10	75	123.00	124.07
13	0	0	0	2.0	7	67	203.00	206.20
14	0	0	0	2.0	7	67	205.00	206.20
15	0	0	0	2.0	7	67	205.00	206.20
16	0	0	0	2.0	7	67	212.00	206.20
17	0	0	0	2.0	7	67	206.00	206.20

Table 2 ANOVA statistics for fitted model for α-L- arabinofuranosidase produced by *P. janthinellum*

	F-value	p-value	
		Prob.>F	Significant
Source			
Model	236.43	<0.0001*	
A- (NH4)2SO4(%)	431.8	<0.0001*	
B-pH	91.01	<0.0001*	
C- Moisture level (%)	125.96	<0.0001*	
A^2- (NH4)2SO4(%)	792.61	<0.0001*	
B^2-pH	262.13	<0.0001*	
C^2- Moisture level (%)	184.25	<0.0001*	
A×B	99.01	<0.0001*	
A×C	0.23	0.6438	
B×C	15.31	0.0058	
R^2	99.67		
Adj R^2	99.25		
Lack of fit	0.27	0.1066	non-significant

* signficant at p<0.05,

not-signficant at p<0.05



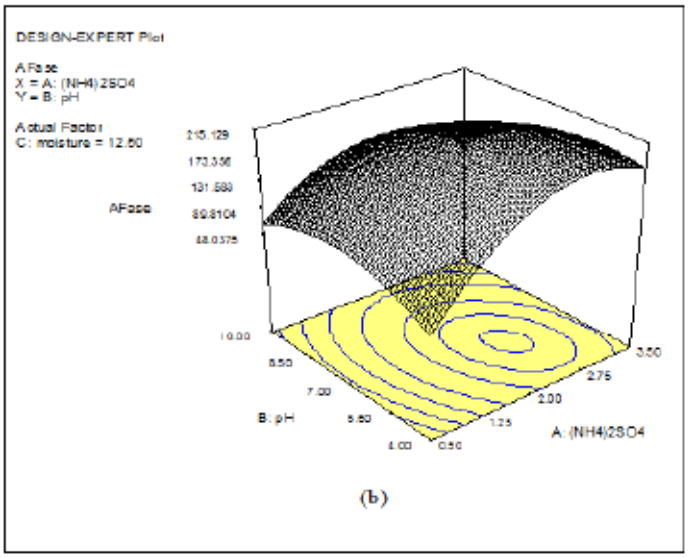


Figure 3 a) 3D contour plot showing the effect of interaction between a) pH and moisture level and b) ammonium sulphate and pH on α-L-arabinofuranosidase (AFase) production by *P. janthinellum*.

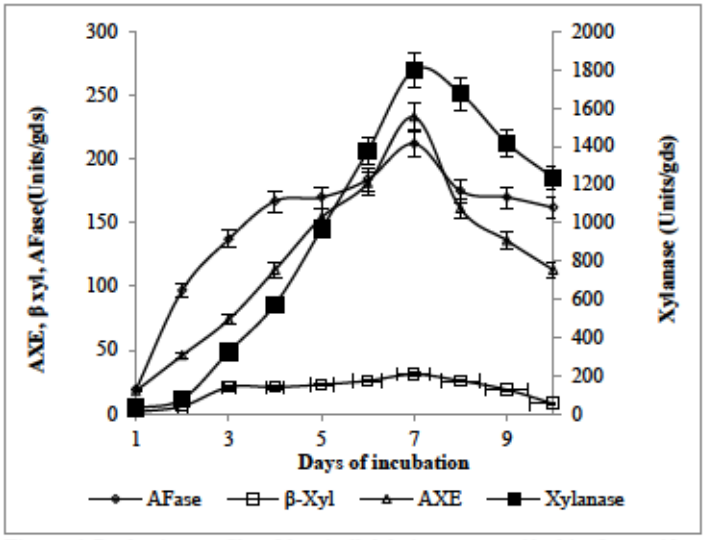


Figure 4 Production profile of hemicellulolytic enzymes (Arabinofuranosidase (AFase), β-xylosidase (β-xyl), Acetylxylan esterase (AXE) and Xylanase) produced by *P. janthinellum* under optimized conditions. Error bars indicate SE @ 5% level.

Table 3 Hemicellulolytic enzymes produced by different fungal strains under SmF and SSF

Fungi	Xylanase	β-xylosidase	α-L-ARAF	AXE	FAE	Reference
SmF (U/ml)						
Penicillium janczewskii	15.2	0.16	0.67	-	-	Terrasan et al .2010
Talaromyces thermophilus	22	1.2	0.85	-	-	Guerfali et al. 2011
Penicillium oxalicum	115.2	0.09	0.04	-	-	Liao et al. 2012
Aspergillus niger (Endophyte)	21.34	0.0	0.21	-	-	Robl et al. 2013
Talaromyces wortmanni (Endophyte)	4.85	2.85	0.91	-	-	Robl et al. 2013
SSF(U/gds)						
Penicillium brasilianum	709	-	3.5	-	1.5	Panagiotou et al. 2006
Aspergillus niger NRRL 328	950	-	-	-	-	Montibeller et al. 2014
Penicillium janthinellum	1800	31	212	233	27	Present work

Purification and characterization of AFase

AFase from *P. janthinellum* was purified to homogeneity using ultrafiltration, ion exchange, hydrophobic and gel chromatography. The purified enzyme exhibited specific activity of 9 $\mu mol^{-1}mg$ $protein^{-1}$ corresponding to 4.5 fold purification. The purified enzyme had a molecular mass and isoelectric point of 64 KDa and 3.8, respectively (Fig 5a & 5b).

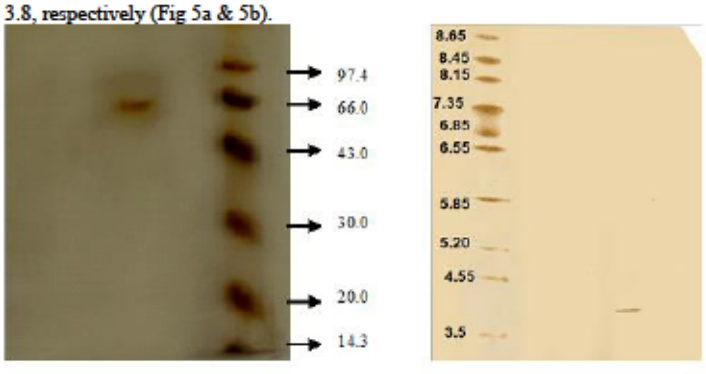


Figure 5 a) SDS-PAGE of purified α-L-arabinofuranosidase from *P. janthellium*. Lane M: standard protein markers of increasing molecular mass: soyabean trypsin inhibitor (20KDa); bovine serum albumin (43KDa); bovine serum albumin (66KDa); phosphorylase (97.4KDa), Lane 1: purified α-L-arabinofuranosidase. b) Isoelectric focusing of purified α-L-arabinofuranosidase (E). Lane M: standard pI markers(sigma). Lane1: purified α-L-arabinofuranosidase.

Temperature, pH optima and Stability

Purified AFase was optimally active at 50° C (Fig 6a). Further increase in temperature resulted in appreciable decline in its activity. The AFase from *P. janthinellum* was optimally active at pH 5.5 (Fig 6b). The AFase retained 98% of its activity after 2 hours at 50° C. (Fig. 6c). Furthermore, the AFase was appreciably stable showing half- life of more than 150 min at 50° C and pH 6.0 (Fig 6c). Table 4 summarizes the comparative properties of AFase from different fungal strains.

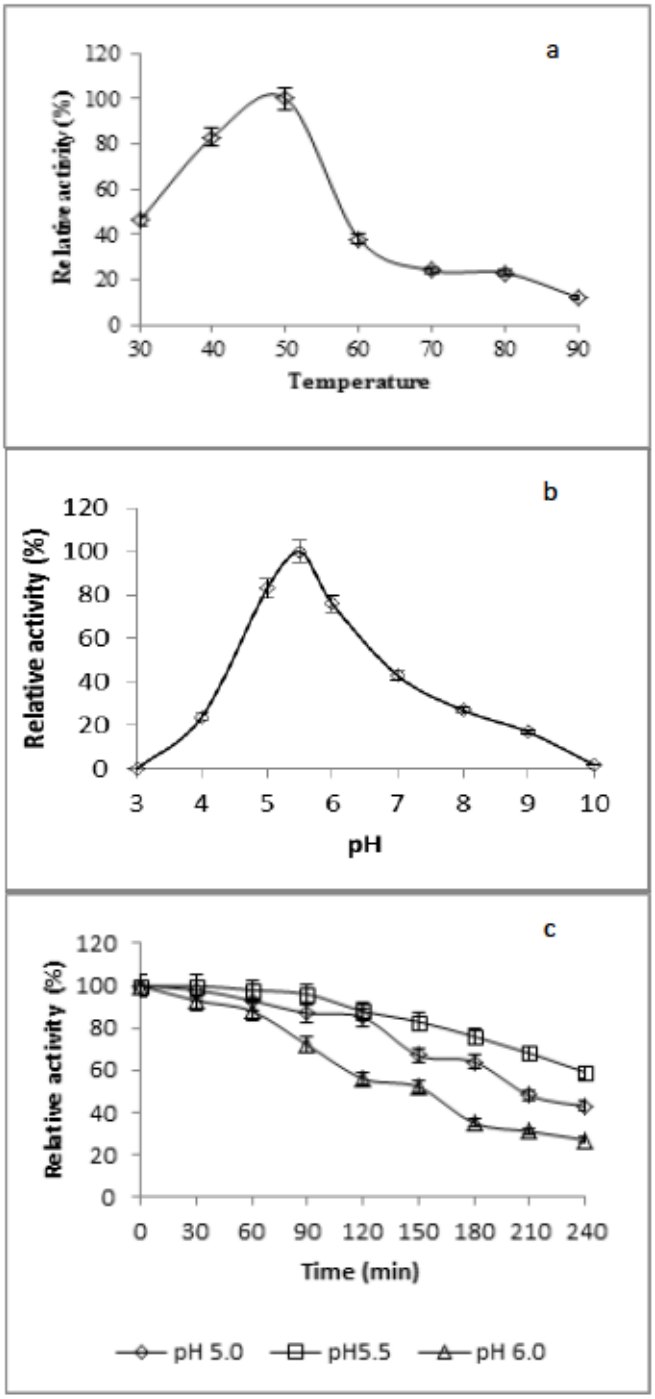


Figure 6 a) Effect of temperature. b) pH on α-L-arabinofuranosidase activity. c) Stability of α-L-arabinofuranosidase at pH 5.0, 5.5 and 6.0 at 50 °C as a function of time. Error bars indicate SE @ 5% level

Table 4 Properties of α-L-arabinofuranosidases (AFase) from different fungal strains

Microoganism	MW (kDa)	T_{opt} (°C)	pH_{opt}	Family	References
Fungi					
Penicillium canescens	*60* *70*	-	-	*54a* *51*	Gusakov et al. 2013
Penicillium janczewskii	-	*25*	*6.0*	-	Terrasan et al. 2010
Penicillium purpurogenum (ABF1)	*58*	50	4.0		De Ioannes et al.2000
Penicillium purpurogenum (ABF2)	*70*	*60*	*5.0*	*51*	Fritz et al.2008
Penicillium crysogenum	*52*	-	*3.3-5.5*	-	Sakamota and Kawasaki.2003
Penicilium brasilianum	-	25.5	6.0	-	Panagiotou et al.2005
Chaetomium sp.	*52.9*	*65*	*5.0*	-	Yan et al. 2012
Humicola insolens	-	*40*	*6.0*	*43*	Sorensen et al.2006
Penicillium janthinellum	64	50	5.5	54	Present work

Effect of metal ions

The activity of purified enzyme was positively modulated in the presence of Fe^{+3} (Fig 7). The activity of purified AFase was inhibited in the presence of Zn^{+2} and Cu^{+2}. N-bromosuccinate resulted in complete loss of enzyme activity.

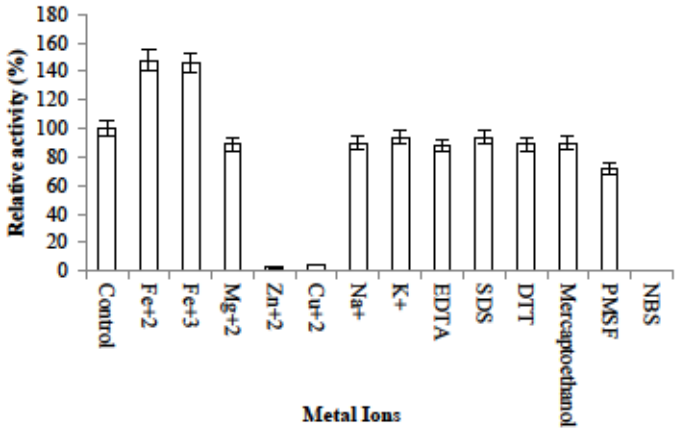


Figure 7 Effect of metal ions and other chemical reagents (10mM each) on the activity of α-L-arabinofuranosidase from *P. janthinellum*. Error bars indicate SE @ 5% level

Substrate specificity and Enzyme Kinetics

The AFase from *P. janthinellum* was maximally active against pNP-α-L-arabinofuranoside (pNPA) but was not able to degrade other pNP substrates. The purified enzyme did not show activity against p-nitrophenyl 2-*O* and 5-*O*-feruloyl α-L-arabinofuranoside. The AFase from *P. janthinellum* was more active on arabinoxylan (Wheat and Rye) than on debranched arabinan. AFase did not catalyse the hydrolysis of birchwoodxylan, CMC (low and high viscosity), and avicel. The purified AFase exhibited K_m and V_{max} of 0.4mM and 260μmol min^{-1}mg $protein^{-1}$against pNPA respectively.

Hydrolysis studies

Thin layer chromatography (TLC) and HPLC analysis of hydrolysis products of rye arabinoxylan, wheat arabinoxylan and debranched arabinan (Fig 8) indicated that AFase from *P. janthinellum* releases L-arabinose as the main sugar. The HPLC based quantification showed that hydrolysis with purified AFase (3.0 units/g substrate) released appreciable amounts of arabinose from rye arabinoxylan (29.5 mg/g substrate) followed by wheat arabinoxylan (13.4 mg/g substrate), and debranched arabinan (3.8 mg/g substrate mg/g) indicating exo-activity of AFase.

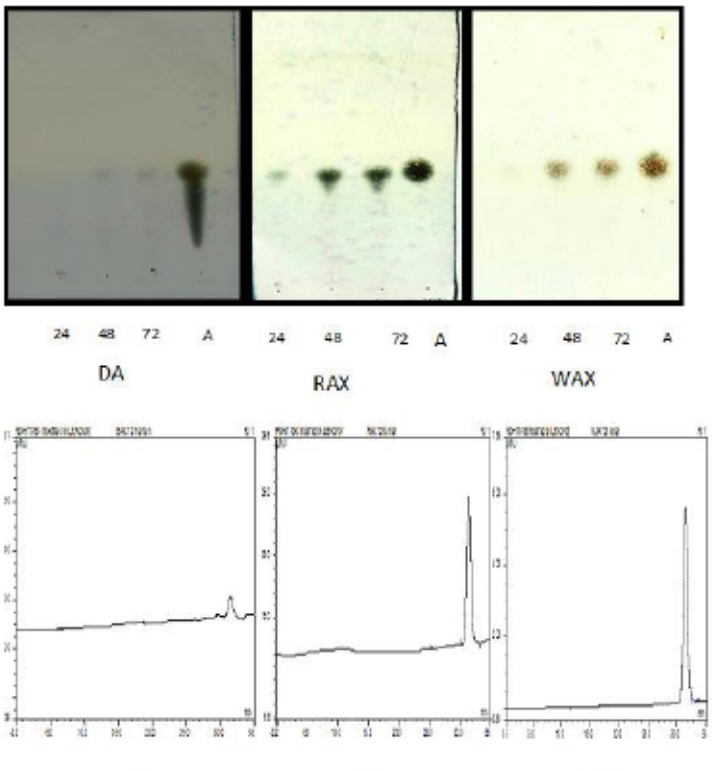


Figure 8 Thin layer chromatography and HPLC showing hydrolysis products obtained by action of α-L-arabinofuranosidase from *P. janthinellum* on a) RAX, b) WAX and c) DA

DISCUSSION

P. janthinellum strain grown on solidified medium containing rice straw and wheat bran as substrates produced high levels of AFase along with other hemicellulosic components (xylanase, β-xylosidase and acetylxylan esterase). The crude enzyme extract resolved by 2 Dimensional electrophoresis and its zymogram developed by using MUA as substrate revealed three distinct spots of high molecular weight and acidic pI. The observed multiplicity of AFase may be due proteolysis of the enzyme during processing that has also been observed previously for multi-domain enzymes such as GH5 β mananase (**Takasuka *et al.*, 2014**) or may be due to cross reactivity of β-xylosidase that can recognise MUA as substrate (**Wagschal *et al.*, 2009**). This is the first report for detection of AFase on 2DE gels by zymography techniques. Further the optimized culture conditions were established for enhancing AFase production. The model computed R^2 and adjusted R^2 for AFase production were 0.997 and 0.992. The R^2 value closer to 1 indicates that the model is robust to predict the response (**Babu and Satyanarayana, 1995**).The regression equation obtained after the analysis of variance (ANOVA) gives the level of enzymes produced as a function of initial value of $(NH4)_2SO4$, moisture level and pH. By applying multiple regressions analysis on the experimental data the second order polynomial equation was found to explain the enzyme production (**Jatinder *et al.*, 2006**). The purification and characterization of AFase from *P. janthinellum* were further

investigated. The results after purification suggest that the purified enzyme is a monomer with a molecular mass of 64 KDa, whereas AFase from *Penicillium* strains (*P. purpurogenum*, *P. chrysogenum* and *P. canescens*) showed a molecular weight of 58, 52 and 60 KDa, respectively (**De Ioannes *et al*, 2000**; **Sakamota and Kawasaki, 2003**; **Gusakov *et al*., 2013**) indicating diversity of AFase produced by different *Penicillium* strains which possibly influences their biochemical characterstics (Saha 2000). The maximal activity of the enzyme was observed at 50° C which is in concurrence with AFase from *Fusarium oxysporium* (50 ° C), *A. pullulans* (55 ° C), *T. thermophilus* (55 ° C) and *Clostridium thermocellum* (50 ° C) (**Chacon *et al*., 2004**; **Dewet *et al*., 2008**; **Guerfali *et al*., 2011**; **Ahmed *et al*., 2013**). The AFase from *P. janthinellum* was optimally active at pH 5.5, similar pH profiles have also been observed in AFase purified from *P. chrysogenum, P. purpurogenum and Chaetomium sp.* (**Sakamota and Kawasaki, 2003**; **Fritz *et al*., 2008**; **Yan et al., 2012**). The activity of purified enzyme was positively modulated in the presence of Fe^{+3}, Similar results were previously observed in *Arthrobacter sp*. (**Khandeparker *et al*., 2008**) whereas the addition of metal ions such as Zn^{+2} and Cu^{+2} inhibited the AFase activity significantly, suggesting that it is a thiol-sensitive enzyme because these heavy metals promote the oxidative process with thiol group and affect the native structure of enzyme thus destabilizing the conformational folding of the enzyme or lead to formation of disulfide bonds at irregular positions (**Ohmiya *et al*., 1995**). Inhibition in the presence of Cu^{+2} has also been previously observed (**Sakamota and Kawasaki, 2003**; **Guerfali *et al*., 2011**; **Yang *et al*., 2012**). Complete loss of the activity in the presence of N-bromosuccinate indicates to the role of tryptophan in the active site of the enzyme (**Adsul *et al*., 2009**). The purified enzyme was maximally active against pNP-α-L-arabinofuranoside as substrate however it did not recognize p-nitrophenyl 2-*O* and 5-*O*-feruloyl α-L-arabinofuranoside which are suitable substrates for determination and differentiation of FAE activity (**Mastihubova *et al*., 2010**). The AFase from *P. janthinellum* was more active on arabinoxylan (Wheat and Rye) than on debranched arabinan. Expectedly purified AFase did not recognised methylglucouronyl and acetyl substituted Birchwood xylan as substrate. The substrate specificity of *P. janthinellum* AFase is similar to GH family 54 AFase from *P. purpurogenum* and *P. funiculosum* (**De Ioannes *et al*., 2000**; **Guais *et al*., 2010**) that catalyzes the removal of α-L-arabinofuranosyl residues from singly substituted xylopyranosyl residues (**Sorensen *et al*., 2006**). The observed K_m (0.4mM) is lower than that of AFase from *P. purpurogenum* (1.23mM), *Aureobasidium pullulans* (3.7mM), *T. thermophilus* (0.77mM) and *Chaetomium* sp. CQ31(1.43mM) (**De Ioannes *et al*., 2000**; **Dewet *et al*., 2008**; **Guerfali *et al*., 2011**; **Yan *et al*., 2012**). Lower k_m indicates much higher affinity for the substrate. Furthermore the purified AFase showed higher rate of catalysis (V_{max}) when compared to AFase from *Chaetomium sp*. (**Yan *et al*., 2012**). The AFase hydrolyzed rye arabinoxylan, wheat arabinoxylan and debranched arabinan releasing arabinose, similar results have previously been observed in AFase of *Clostridium sp*. by (**Ahmed *et al*., 2013**) whereas GH54 AFase from *A. pullulans* (**Dewet *et al*., 2008**) was able to release arabinose from arabinoxylans but not from debranched arabinan. Debranched arabinan is mainly comprised of chain of α, 1,5-linked arabinofuranosyl residues whereas, arabinoxylans possess a xylan backbone that is substituted with arabinose and acetyl moieties through α,1,2 and α,1,3 linkages. The enzyme used in the present study seems to cleaves arabinose from α1, 2 and α1,3 linkages. The above results also indicate the exo-activity of AFase which has immense potential in bioconversion of agro-residues to value added products (**Guerfali *et al*., 2010**).

CONCLUSION

The study reports *P. janthinellum* as highly efficient source of AFase as well as other components of hemicellulolytic enzyme system. By employing response surface methodology optimal culture conditions for AFase production were established which were higher than reported earlier. Secretome analysis revealed *P. janthinellum* α-L-arabinofuranosidase belonging to family 54. This is the first report on identification of α-L-arabinofuranosidase on 2DE gels by zymography. Also purified AFase efficiently hydrolyses rye and wheat arabinoxylan to arabinose. Thus this AFase can be of great importance to food and bioconversion industry.

Conflict of interest: The author confirms that this article content has no conflict of interest.

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DIVERSITY OF MYCOBIOTA ASSOCIATED WITH ONION (*ALLIUM CEPA* L.) CULTIVATED IN ASSIUT, WITH A NEWLY RECORDED FUNGAL SPECIES TO EGYPT

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ABSTRACT

The goal of this study was to characterize diversity of fungal biota in soil, roots and green leaves of onion plant. Seventy- nine fungal species belonging to 32 genera were isolated from soil (29 genera and 72 species), rhizosphere (25 and 52), rhizoplane (24 and 38), phyllosphere (17 and 41) and phylloplane (17 and 35) on PDA medium at 19° and 28°C. The number of fungal genera and species in soil was higher than those on roots and leaves, while those on the surface of roots (rhizosphere) or leaves (phyllosphere) were higher than those adhering to roots (rhizoplane) or leaves (phylloplane). *Aspergillus* (*A. niger* and *A. terreus*), followed by *Penicillium* (*P. funiculosum* and *P. chrysogenum*), *Rhizopus* (*R. stolonifer*) and *Fusarium* (*F. oxysporum*) were the most common fungi. A new record species is reported for the first time to Egypt namely, *Zopfiella latipes* (from phylloplane of onion).

Keywords: Soil, onion, *Allium cepa*, rhizosphere, rhizoplane, phyllosphere, phylloplane, *Zopfiella latipes*

INTRODUCTION

Onion (*Allium cepa* L., Alliaceae) is one of the main important and oldest vegetable crops grown in Egypt. Onion although primarily grown for food, is also used in traditional medicine, including the treatment of chicken pox, the common cold, influenza, measles and rheumatism. Antimicrobial characteristics of the *Allium* are related to the effect of sulfur compounds produced in its tissues. Onion may help to prevent arteriosclerosis and other cardiovascular diseases (**Schwartz and Mohan, 2007**). The phyllosphere of plants is a dynamic ecosystem inhabited by specific bacteria and fungi. Their activity is related to various interactions between the biotic and abiotic factors of the environment (**Behrendt *et al.*, 1997, 2002**). Saprotrophic leaf surface fungi perform key ecological roles in the plant and aerial plant surfaces provide a suitable habitat for epiphytic microorganisms, which are influenced by the nutrients present on the leaf surfaces (**Tyagi *et al.*, 1990; Abdel-Hafez *et al.*, 2015**). Phylloplane provides a suitable habitat for the growth of microorganisms which can compete with the pathogen for nutrients and inhibit pathogen multiplication by secreting antibiotics or toxins (**Yadav *et al.*, 2011; Thakur and Harsh, 2014**). Several studies were carried out to characterize the mycobiota of root surface and soil adhering the roots of onion plants. *Penicillium*, *Aspergillus*, *Trichoderma* and *Cladosporium* were detected from the rhizosphere of onion seedling (**Lyndsay, 1973**). In another study, 5 Zygomycetous species, 9 Ascomycetous species and 59 Hyphomycetous species were isolated from the rhizosphere of *Allium cepa* (**Bertoldi *et al.*, 1978**). **Abdel-Sater (2001)** identified twenty fungal species from leaf surfaces of onion plant of which *Alternaria alternata, Aspergillus niger, A. sydowii, A. versicolor, Cladosporium herbarum, Cochliobolus lunatus, Pleospora herbarum, Setosphaeria rostrata* and *Ulocladium botrytis* were the most prevalent. **Montes-Belmont *et al.* (2003)** isolated also *Fusarium, Rhizoctonia, Curvularia, Phoma, Alternaria, Sclerotium, Bipolaris, Aspergillus, Rhizopus* and *Penicillium* from onion nurseries. *Fusarium culmorum, Penicillium* and *Colletotrichum circinans* were also reported as pathogens for onion bulbs and it is recommended to use eco-friendly root and leaf surface microorganisms to manage plant pathogens (**Abo-Shady *et al.*, 2007; Soria *et al.*, 2012; Abo-Elyousr *et al.*, 2014**). Hence, it is necessary to determine the fungal populations in the soil, root and leaf regions which could have positive or negative impact on onion growth and development. This study aimed to provide comprehensive information on the fungi associated with soil, rhizosphere, rhizoplane, phyllosphere and phylloplane of onion (Giza 6) during the period from planting till harvesting.

MATERIAL AND METHODS

Collection of Samples

Two localities in Assiut Governorate were selected for the present study; Botanical Garden of Faculty of Science, Assiut University and Refa Village (12 Km south of Assiut city). Samples were collected monthly during the growing season which extended from September 2005 to April 2006.

Soil samples: Twenty-six soil samples were collected at a depth of 5 inches, put in sterilized polyethylene bags and mixed thoroughly and transferred directly to the laboratory (**Johnson *et al.*, 1959**).

Root samples: Onion roots (20 samples) were uprooted from the soil and shaken gently to collect the adhering soil. Then the roots and soil were placed separately in sterilized polyethylene bags and transferred to laboratory.

Samples of green leaves: For determination of phyllosphere and phylloplane fungi, 20 samples of green tubular leaves of onion were collected by cutting using sterilized scissors and packed directly into polyethylene bags and transferred to laboratory.

Isolation of Fungi

Soil borne fungi: Potato Dextrose Agar medium (PDA) supplemented with rose-bengal (0.067g/l) and chloramphenicol (0.25 g/l) as bacteriostatic agents (**Smith and Dawson, 1944; Booth, 1971**) was used. The dilution-plate method was employed to determine soil fungi (**Johnson *et al.*, 1959; Moubasher *et al.*, 1977**). One ml of the desired dilution was transferred directly into each of sterilized 9 cm diameter Petri dishes, then, ~20ml of PDA were poured in each plate and stirred gently for homogenous distribution of soil suspension. The plates were incubated either at 19°C and 28°C for 7 days (five replicates for each sample). The developing colonies were enumerated and identified.

Rhizosphere fungi: The dilution plate method was used to isolate rhizosphere fungi. The PDA plates were incubated at either 19°C or 28°C (five replicates for each sample) for 7 days during which the developing fungi were counted and identified.

Rhizoplane fungi: The previously uprooted roots of onion plant were subjected for a series of washings with sterilized distilled water, dried, cut into equal segments (1 cm long). Five segments were placed on the surface of the PDA medium plates. The plates were incubated at 19ºC and 28ºC (5 replicates for each sample) for 7 days during which the developing colonies were counted and identified.

Phyllosphere fungi: Green leaves of onion were cut into segments (1 cm diam each). Twenty g of these segments were placed in sterile conical flasks containing 100 ml sterile distilled water and were shaken for 20 minutes. Final desired dilution (1/500) was prepared. One ml of the final dilution was transferred into sterilized Petri dish, and then 10-15 ml of melted PDA medium was poured and shaken gently. The plates were incubated at 19ºC and 28ºC for 7 days (5 replicates for each sample). Developing colonies were identified and counted.

Phylloplane fungi: The previous segments of onion green leaves were washed several times with sterilized distilled water. Then they were dried thoroughly between sterilized filter paper. Five segments (1 cm diam.) were placed on the surface of PDA plate. Five replicates were used for each sample and the plates were incubated at 19° and 28ºC. The developing fungi were counted and identified.

Identification of fungi

The fungal colonies were identified based on macro- and microscopic characters following **Raper and Fennell (1965)**, for *Aspergillus* species; **Ellis (1971, 1976)**, for Dematiaceous Hyphomycetes; **Booth (1971)**; **Leslie and Summerell (2006)**, for *Fusarium* species; **Pitt (1979)**, for *Penicillium* species; **Moubasher (1993)**, **Pitt and Hocking (1997)** and **Domsch *et al.* (2007)** for fungi in general.

Statistical analysis

Hierarchical clustering analysis using free online software statistical analysis (www.wessa.nit.com) was used and Detrended Correspondence Analysis (DCA) was performed using Canoco 4.5 **(Ter Braak and Šmilauer, 1998)** to ordinate sources based on their fungal composition.

RESULTS AND DISCUSSION

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RESULTS AND DISCUSSION

Seventy-nine species belonging to 32 genera were identified from soil (58 species and 25 genera), rhizosphere (47 and 23), rhizoplane (30 and 16), phyllosphere (37 and 15) and phylloplane (28 and 14) on PDA medium at 19°C. While lower number of genera (insert the number) and species (insert the number) were recovered at 28°C from soil (26 genera, 60 species), rhizosphere (19 genera, 40 species), rhizoplane (13 genera, 28 species), phyllosphere (14 genera, 36 species) and from phylloplane (12 genera, 25 species) (Table 1).

Fungi isolated from soil samples

Seventy-two species appertaining to 29 genera were isolated from soil cultivated with onion plant on PDA plates incubated at 19° and 28°C. *Aspergillus*, *Penicillium*, *Cochliobolus*, *Fusarium* and *Rhizopus* were the most common genera at both 19° and 28°C. They were recorded in 65.38 - 100% of total samples tested (Table 1). The gross total fungal count was higher at 19°C than at 28°C as shown in table (1).

Aspergillus was represented by 9 and 8 species comprising 33.30 and 50.21% of total fungi at 19° and 28°C respectively. *A. niger* and *A. terreus* were isolated in high frequencies (ranging between 73.08% and 100% of total samples tested). On the other hand, *A. sydowii* was recorded only at 19°C with 11.54% frequency of occurrence, while two species (*A. carbonarius* and *A. oryzae*) were isolated only at 28°C. *A. niger* was reported as an abundant soil-borne fungus and may be a source of black mould of onion **(Tyson and Fullerton, 2004).**

Penicillium occurred in 84.62% and 88.46% of total samples at 19° and 28°C respectively. It was represented by 12 species of which, *P. funiculosum* was the most common, followed by *P. chrysogenum* at 19°C and *P. oxalicum* at 28°C. *P. islandicum* and *P. mirabile* were detected only at 19°C, but *P. pinophilum* was isolated at 28°C.

Cochliobolus (3 species), with its predominant species *C. spicifer*, was detected in 76.92% of total samples. *C. hawaiiensis* was isolated from 3.85% of total samples at only 28°C (Table 1).

Fusarium was represented by 6 and 5 species at 19° and 28°C respectively. *F. oxysporum* was the most common species, followed by *F. solani*. *F. equiseti, F. tricinctum* and *F. xylarioides* were isolated only at 19°C, and *F. oxysporum* var. *redolens* and *F. subglutinans* were isolated rarely at 28°C only.

Rhizopus stolonifer appeared in 84.62% and 65.38% of total samples, accounting 6.56% and 6.43% of total fungi at 19° and 28°C respectively (Table 1). The remaining genera (20 genera at 19°C and 21 genera at 28°C) were recorded in moderate or low frequency of occurrence (Table 1).

Several fungal genera and species were commonly isolated from soil in Egypt **(Abdel-Hafez *et al.*, 2000; Zohri *et al.* 2014; Elkhateeb *et al.*, 2016).**

Rhizosphere fungi

Fourty-seven and 40 fungal species belonging to 23 and 19 genera constituting 679945 and 572980 cfu/g were isolated from 20 rhizosphere samples on PDA at 19° and 28°C respectively (Table 1). The number of species in the rhizosphere (52 species) was less than that in the soil away from it (72 species). This is probably due to that exudates secreted from onion roots preventing non-rhizosphere fungi to gain access to the rhizosphere. Our results are in harmony with those of **Sule and Oyeyiola (2012)**, while in contrast with those of **Mehrotra and Kakkar (1972)** who recorded greater number of fungi in the rhizosphere than in the soil. *Aspergillus* (8 species) comprised 54.49% and 63.32% of total fungi at 19° and 28°C respectively. *A. niger* was the predominant species followed by *A. terreus* and *A. versicolor* were isolated in moderate frequency at 28°C and in low frequency at 19°C. *A. flavipes* was isolated only at 28°C and *A. niveus* at 19°C only (Table 1). *Penicillium* was represented by 10 and 9 species matching 14.75 and 15.18% of total fungi at 19° and 28°C respectively. *P. funiculosum* was isolated moderately, while the remaining *Penicillium* species occurred in low frequency. The results showed that *P. chrysogenum*, *P. mirabile* and *P. pinophilum* were recorded at 19°C only, whereas, *P. citrinum* and *P. islandicum* were isolated at 28°C only. *Rhizopus stolonifer* occurred in 65% of the total samples tested, matching 6.26% and 6.75% of total fungi at 19° and 28°C respectively. Our results indicated that, *Acrophialophora fusispora*, *Botrytis cinerea*, *Humicola grisea*, *Rhizoctonia solani*, *Setosphaeria rostrata* and *Stemphylium botryosum* were isolated at 19°C only, while, *Cunninghamella echniulata* and *Macrophomina phaseolina* were isolated at 28°C only.

The above species were frequently recovered from rhizosphere and non rhizosphere soils of various plants cultivated in different localities of Egypt as reported by several workers **(Moubasher and Abdel-Hafez, 1986; Abdel-Hafez *et al.*, 1990, 2000; Elkhateeb *et al.*, 2016)**.

Rhizoplane fungi

Thirty and 28 species belonging to 16 and 13 genera were collected from onion roots at both 19° and 28°C respectively on PDA medium (Table 1). The gross fungal count was slightly higher at 28°C (1327 cfu/25 root segments) than at 19°C (1276 cfu/25). It is worthy to mention that, the rhizosphere of onion roots hosted a broader spectrum of species than that of the rhizoplane (53 and 38 species respectively). This is in harmony with the results obtained by **Sule and Oyeyiola (2012)** who isolated 30 and 18 different fungal species as rhizosphere and rhizoplane fungi respectively. Also, **Porras-alfaro *et al.* (2011)** demonstrated that microbial richness in rhizosphere and soil samples was nearly three times greater than the richness described for fungal communities associated with roots of plants at the same site. *Aspergillus* was represented by 5 species, comprising 90% and 100% at 19° and 28°C respectively (Table 1). *A. niger* was the most prevalent species at 19°C and at 28°C. *A. sydowii* was isolated only at 19°C, while *A. ochraceus* was detected only at 28°C. The remaining species were recorded in low or rare frequencies. *Fusarium*, with its dominant species *F. oxysporum*, was represented by 2 and 3 species, comprising 19.98% and 12.51% of total fungi and occurred in 90% and 70% of the samples at 19° and 28°C respectively. *F. equiseti* was isolated rarely at 28°C only. *Fusarium oxysporum* and *F. solani* altogether with other fungal species were isolated from onion roots and bulbs in Northeast of Iran **(Rabiei- Motlagh *et al.*, 2010)**. *Penicillium* (9 and 8 species) and *Rhizopus (R. stolonifer)* were recorded in high frequency at 19° and 28°C. *Botrytis cinerea, Chaetomium globosum, Cunninghamella echinulata, Emericella nidulans, Epicoccum nigrum, Phoma leveillei* and *Rhizoctonia solani* were isolated at 19°C only, but *Acremonium strictum, Macrophomina phaseolina, Setosphaeria rostrata* and *Sordaria fimicola* were isolated at 28°C only (Table 1). Several of these species were infrequently recovered from rhizoplane of some plants cultivated in Egypt **(Moubasher and Abdel-Hafez, 1986; Abdel-Hafez *et al.*, 1990, 2000)**.

Phyllosphere fungi

Fifteen genera and 37 species (at 19°C); 14 genera and 36 species (at 28°C) were recorded as phyllosphere fungi from green leaves of onion (Table 1). The total counts were slightly higher at 19°C (133500 cfu/g green leaves) than at 28°C (131300). *Aspergillus* (8 and 7 species, comprising 33.4% and 38.54% of total fungi at 19° and 28°C respectively), *Cladosporium* (3 species, 23.1% and 21.25% of total fungi), followed by *Penicillium* (10 and 9 species, 10.8% and 16.76% of total fungi) were the most common genera (Table 1). *Cladosporium* spp. are active at low temperature and high humidity and are known as important pathogens to plant leaves **(Kwon *et al.*, 2001)**. Other four fungal species were isolated at 19°C only (*A. oryzae, Gliocladium roseum, P. waksmanii* and *Stachybotrys chartarum*), while 3 species were detected at 28°C only (*C. hawaiiensis, F. verticillioides* and *Mucor circinelloides*).

Abdel-Hafez *et al.* (2015) isolated 58 fungal species belonging to 25 genera as phyllosphere fungi from healthy leaves of onion plant. *Alternaria alternata, Aspergillus niger, A. terreus, Cladosporium cladosporioides, Penicillium funiculosum*, and *Trichoderma harzianum* were recovered in high frequency, while *Aspergillus carbonarius, A. flavipes, Cunninghamella echinulata, Epicoccum nigrum, Humicola grisea, Myrothecium verrucaria, Nigrospora sphaerica, Penicillium citrinum* and other fungi were rarely isolated.

Phylloplane fungi

Fourteen and 12 genera including 28 and 25 species and contributing 811 and 944 cfu/25 leaf segments were isolated at 19° and 28°C respectively (Table 1). *Aspergillus* (7 species) was recorded in all samples matching 36.50% and 49.68% of total fungi at 19° and 28°C respectively. *A. niger* was the most common species, detected in all samples, contributing 28.24% and 40.89% of total fungi at 19° and 28°C respectively. *A. versicolor* was isolated at 19°C only, while *A. ustus* was recorded at 28°C only. The remaining species were infrequent (Table 1). *Penicillium* was isolated in moderate frequency (65% of samples) at 19°C representing 12.59% of total fungi and high frequency at 28°C (13.56% and 70% respectively). It was represented by 7 and 6 species at 19° and 28°C respectively. *P. funiculosum* was isolated moderately occurred in 40% and 55% of total samples, comprising 8.26% and 10.81% of total fungi at 19° and 28°C respectively. It is worthy to mention that, *P. corylophilum, P. fellutanum, P. mirabile* and *P. waksmanii* were recorded at 19°C only, while *P. citrinum, P. duclauxii* and *P. pinophilum* were isolated at 28°C only. The remaining *Penicillium* species (*P. chrysogenum* and *P. oxalicum*) were recovered in low frequency at both 19° and 28°C (Table 1). *Cladosporium* (2 species) and *Alternaria alternata* were isolated in moderate occurrence, while *Rhizopus stolonifer* was isolated in moderate and low frequencies at 28° and 19°C, respectively. In most reports, there is a marked dominance of anamorphic fungi, mostly of ascomycetous affinity, and the main genera found in the phylloplane are *Cladosporium*, *Aspergillus*, *Alternaria*, *Aureobasidium* and *Epicoccum* **(Pereira *et al.*, 2002; Guimarães *et al.*, 2011)**. Results in Table (1) revealed that, *Beauveria bassiana, Macrophomina phaseolina, Phoma leveillei* and *Rhizoctonia solani* were recorded at 19°C only, while *Acremonium strictum* and *Zopfiella latipes* were isolated at 28°C only in rare frequencies. *Acremonium strictum, Alternaria alternate, Aspergillus flavus, A. fumigates, A. niger, A. sydowii, A. terreus, Cladosporium cladosporioides, C. sphaerospermum, Fusarium oxysporum, Gliocladium roseum, Penicillium duclauxii, P. pinophilum, Stemphylium botryosum* and *S. vesicarium* were isolated previously from phylloplane of healthy green leaves of onion **(Abdel-Hafez *et al.*, 2015)**. *Aspergillus niger* and *Cladosporium cladosporioides* were also the most common species of leaf surface of onion as recorded previously by **Abdel- Sater (2001)**.

In the present study, the number of phyllosphere fungi of green leaves (41 species) exceeded that of phylloplane fungi (33 species). This means that about 22% of fungal species are not really inhabitants of the leaf surface, but are deposited from the air. These results are in agreement with those reported by **Abdel- Sater (2001)** and **Abdel-Hafez *et al.* (2015)**, who examined the leaf surface fungi of onion plants and reported that the number of phyllosphere fungi (58 and 20 species respectively) outnumbered those of the phylloplane (25 and 9 respectively). Results of the present study revealed that, the lowest number of genera (17 and 17) and species (41 and 35) was almost isolated from leaves as phyllosphere and phylloplane mycobiota respectively. This is probably because the leaf surface is exposed to rapidly fluctuating temperature and relative humidity, as well as repeated alternation between presence and absence of free moisture content dew. Also, the leaf itself is surrounded by a very thin laminar layer in which moisture emitted through stomata may be sequestered, thereby alleviating the water stress to which epiphytes are exposed **(Lindow and Brandl, 2003)**.

Temperature is one of the limiting factors in fungal growth and spread. In the present study, some fungi were isolated either at 19°C, such as *Humicola grisea, P. mirabile, Rhizoctonia solani*; or at 28°C, such as *Cheatomium brasiliense* and *Zopfiella latipes*. However the optimum temperature for *Rhizoctonia solani* growth ranged between 15 – 30°C **(Orozco-Avitia *et al.*, 2013)**.

In cluster analysis, the 5 sources (soil, rhizosphere, rhizoplane, phyllosphere and phylloplane) and two incubation temperatures (19° and 28°C) were grouped based on total counts of fungal species (Figure 1). The analysis showed that, fungal species isolated from soil at 28°C and rhizosphere at both 19° and 28°C cluster closely together and they are the most similar to fungal community in soil at 19°C. Also, fungal communities isolated from phyllosphere at both 19° and 28°C were clustered together in the same group (C). The cluster analysis in figure (2) was used to compare different sources according to their number of genera and species. The cluster classify sources into four groups of which groups A and B are closely related, while, fungal genera and species isolated from soil at both 19° and 28°C (group D) showed the least similarity with those isolated from other sources (Figure 2).

Figure 3a exhibited the distribution of genera is significantly different, for example *Acrophialophora* and *Setosphaeria* were closely related and showed significantly difference from *Alternaria*, *Fusarium* and *Trichoderma*. On the other hand, figure (3b) shows differences in composition of fungal genera isolated from different sources at 28°C using detrended correspondence analysis (DCA). Interestingly, *Macrophomina* and *Rhizoctonia* are closely related and significantly different from *Alternaria*, *Fusarium* and *Sordaria*.

Table 1 Percentage total counts (%TC) and percentage frequency (%F) of fungi isolated from soil, rhizosphere, rhizoplane, phyllosphere and phylloplane of onion plants on PDA medium at both 19 and 28°C. (n: the number of samples collected)

Fungal taxa	Soil (n = 26)				Rhizosphere (n = 20)				Rhizoplane (n = 20)				Phyllosphere (n = 20)				Phylloplane			
	19°C		28°C		19°C		28°C		19°C		28°C		19°C		28°C		19°C		28°C	
	%TC	%F	%TC	%F	%TC	%F	%TC	%F	%TC	%F	%TC	%F	%TC	%F	%TC	%F	%TC	%F	%TC	%F
Acremonium strictum W. Gams	0.1	14.5	0.8	19.2	0.99	15.00	0.74	10.00	-	-	0.08	5.00	0.1	5	0.2	5.0	-	-	0.3	5.0
Acrophalophora fusispora (Saksena) Samson	-	-	0.1	11.5	0.10	5.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alternaria alternata (Fries) Keissler	1.9	57.7	2.5	61.5	1.21	40.00	0.61	30.00	7.13	45.00	3.69	35.00	2.1	25	2.9	35.0	7.3	35.0	4.8	35
Aspergillus P. Micheli ex Link	93.5	100.0	50.2	100.0	54.49	100.00	63.32	100.00	25.63	90.00	39.64	100.00	33.4	100	38.5	95.0	36.5	100	49.7	100
A. carbonarius (Bainier) *et al.*	-	-	0.2	11.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. flavipes (Bainier & Sartory) Thom & Church)	-	-	-	-	-	-	0.12	5.00	-	-	-	-	-	-	-	-	-	-	-	-
A. flavus Link	32.2	34.6	2.0	46.2	0.78	20.00	0.65	10.00	2.19	10.00	2.26	15.00	2.4	25	2.1	25.0	4.3	20	4.3	25
A. fumigatus Fresenius	0.8	14.5	0.1	7.7	5.16	25.00	0.31	10.00					0.3	10	0.5	10.0	0.2	5.0	0.4	10.0
A. niger van Tieghem	24.1	100.0	34.9	100.0	44.93	95.00	55.70	95.00	21.71	90.00	35.57	100.00	27.1	100	29.9	95.0	28.2	100	40.7	100
A. niveus Blochwitz	-	-	-	-	0.26	5.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. ochraceus Wilhelm	24.2	19.2	0.2	19.2	0.47	30.00	0.07	5.00	-	-	0.68	10.00	2.1	25	4.2	25.0	1.7	5	3.0	15
A. oryzae (Ahlburg) Cohn	-	-	0.1	7.7	-	-	-	-	-	-	-	-	0.1	5	-	-	-	-	-	-
A. sydowii (Bainier & Sartory) Thom & Church	0.2	11.5	-	-	0.06	5.00	0.37	20.00	0.16	5.00	-	-	0.1	5	0.2	5.0	0.2	5	0.5	10
A. terreus Thom	5.6	73.1	12.0	84.6	1.96	30.00	3.81	55.00	1.41	20.00	0.75	15.00	0.9	15	1.4	30.0	1.6	20	0.5	15
A. ustus (Bainier) Thom & Church	5.6	3.8	0.0	3.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	5
A. versicolor (Vuillemin) Tiraboschi	0.8	30.8	0.8	30.8	0.88	30.00	2.29	50.00	0.16	5.00	0.38	15.00	0.3	5	0.3	5.0	0.1	5	-	-
Beauveria bassiana (Balsamo) Vuillemin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	5	-	-
Botryotrichum piluliferum Saccardo & Marchal	0.8	14.5	0.0	3.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Botrytis cinerea Persoon	0.5	14.5	-	-	2.49	15.00	-	-	0.39	10.00	-	-	9.5	30	1.8	25.0	11.8	35	2.0	15
Chaetomium Kunze	0.6	14.5	0.1	3.8	0.2	15.0	0.1	5.0	0.2	5.0	-	-	-	-	-	-	-	-	-	-
C. brasiliense Bat. & Pontual	-	-	0.1	3.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. globosum Kunze	0.6	14.5			0.2	15.0	0.1	5.0	0.2	5.0	-	-	-	-	-	-	-	-	-	-
Cladosporium Link	33.4	61.5	5.4	57.7	9.0	60.0	3.9	30.0	0.6	10.0	0.2	5.0	23.1	70	21.2	80.0	10.9	50	9.9	45
C. cladosporioides (Fresenius) de Vries	20.2	46.2	4.4	34.6	5.9	45.0	2.4	20.0	0.6	10.0	0.2	5.0	14.2	60	13.8	75.0	7.2	40	8.1	45
C. herbarum (Pers.) Link ex S. F. Gray	8.1	14.5	0.5	11.5	0.8	10.0	0.5	5.0	-	-	-	-	1.0	15	2.0	25.0	-	-	-	-
C. oxysporum Berkeley & Curtis	-	-	-	-	0.3	5.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. sphaerospermum Penzig	5.1	30.8	0.5	19.2	1.9	20.0	0.9	15.0	-	-	-	-	7.9	35	5.5	15.0	3.7	15.	1.8	10
Cochliobolus Drechsler	11.3	76.9	6.4	76.9	1.7	45.0	2.0	45.0	4.0	30.0	5.5	40.0	1.9	25	1.3	30.0	-	-	-	-
C. hawaiiensis Alcorn			0.0	3.8	0.1	5.0					0.3	5.0			0.1	5.0	-	-	-	-
C. lunatus R. Nelson & Haasis	6.1	42.3	1.2	50.0	0.3	20.0	0.3	15.0	1.6	15.0	2.6	25.0	0.4	15	0.3	10.0	-	-	-	-
C. spicifer Nelson	5.3	73.1	5.1	76.9	1.3	25.0	1.8	40.0	2.4	30.0	2.6	25.0	1.5	30	0.9	20.0	-	-	-	-
Cunninghamella echniulata (Thaxter) Thaxter	4.6	3.8	0.0	7.7			0.1	5.0	0.3	10.0	-	-					-	-	-	-
Emericella Berkeley & Broome	1.7	38.5	3.2	61.5	0.1	5.0	0.1	5.0	0.2	5.0	-	-	0.3	10	0.2	5.0	-	-	-	-
E. lata Subramanian	0.8	11.5	0.1	11.5	-	-	-	-	-	-	-	-					-	-	-	-
E. nidulans (Eidam) Vuillemin	0.3	23.1	1.7	50.0	-	-	-	-	0.2	5.0	-	-	0.3	10	0.2	5.0	-	-	-	-
E. rugulosa (Thom & Raper) Benjamin	0.6	30.8	1.4	34.6	0.1	5.0	0.1	5.0	-	-	-	-	-	-	-	-	-	-	-	-
Epicoccum nigrum Link	0.6	15.4	0.1	7.7	-	-	-	-	0.4	5.0	-	-	0.4	15	0.3	10.0	-	-	-	-
Eurotium amstelodami Mangin	-	-	0.1	7.7	-	-	-	-			-	-	-	-	-	-	-	-	-	-
Fusarium Link	13.5	76.9	8.2	69.2	5.3	50.0	2.2	35.0	20.0	90.0	12.5	70.0	2.7	25	3.1	40.0	3.7	25	2.8	30
F. chlamydosporum Wollenweber & Reinking	4.5	3.8	0.1	3.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F. equiseti (Corda) Saccardo	0.1	3.8	-	-	-	-	-	-	-	-	0.4	15.0	-	-	-	-	-	-	-	-
F. oxysporum Schlechtendal	4.0	69.2	6.3	61.5	5.3	50.0	1.7	35.0	18.9	90.0	11.4	70.0	1.6	20	1.8	25.0	3.7	25	2.8	30
F. oxysporum var redolens (Wollenw.) Gordon	-	-	0.0	3.8													-	-	-	-
F. solani (Martius) Saccardo	4.3	19.2	1.8	42.3	0.1	5.0	0.5	5.0	1.1	15.0	0.8	15.0	1.0	10	1.2	25.0	-	-	-	-
F. subglutinans (Wollenweber & Reinking) Nelson *et al*	-	-	0.0	3.8	-	-	-	-	-	-	-	-	-	-			-	-	-	-
F. tricinctum (Corda) Saccardo	0.5	3.8	-	-	-	-	-	-	-	-	-	-	-	-			-	-	-	-
F. verticillioides (Saccardo) Nirenberg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	5.0	-	-	-	-
F. xylarioides Steyaert	0.1	3.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gliocladium roseum Bainier	0.2	15.4	0.0	3.8	0.1	5.0	0.1	5.0	-	-	-	-	0.4	10	-	-	0.4	10	0.1	5
Humicola grisea Traaen.	0.2	3.8	-	-	0.0	5.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Macrophomina phaseolina (Tassi) Goidanch	0.0	3.8	-	-	-	-	0.1	5.0			0.2	5.0	-	-	-	-	0.2	5	-	-
Mucor circinelloides van Tieghem	0.0	14.5	0.1	7.7	0.6	30.0	1.0	15.0	0.7	10.0	3.5	15.0	-	-	1.4	10.0	-	-	-	-
Mycothecium Tode	5.3	42.3	2.3	38.5	1.1	10.0	1.2	10.0	-	-	-	-	-	-	-	-	-	-	-	-
M. roridum Tode	2.9	11.5	0.8	7.7	0.7	5.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M. verrucoria (Albertini & Schweinitz) Ditmar	2.4	34.6	1.5	34.6	0.5	5.0	1.2	10.0	-	-	-	-	-	-	-	-	-	-	-	-
Nigrospora sphaerica (Saccardo) Mason	2.2	11.5	0.2	11.5	0.1	5.0	0.0	5.0	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium Link	25.2	84.6	9.7	88.5	14.7	65.0	15.2	65.0	17.2	65.0	20.6	75.0	10.8	75	16.8	75.0	19.0	65	13.6	70
P. chrysogenum Thom	9.7	26.9	1.2	11.5	0.4	15.0	-	-	0.2	5.0	-	-	0.4	15	1.3	30.0	0.1	5	0.5	10
P. citrinum Thom	1.4	14.5	0.2	7.7	-	-	0.1	5.0	0.2	5.0	-	-	-	-	-	-	-	-	0.1	5
P. corylophilum Dierckx	0.1	3.8	0.1	11.5	0.2	10.0	0.2	15.0	0.3	5.0	0.3	5.0	0.3	10	0.3	10.0	0.7	10		
P. decumbens Thom	0.2	3.8	0.9	15.4	2.5	15.0	0.3	5.0	-	-	-	-	0.4	5	0.6	10.0				
P. duclauxii Delacroix	0.2	3.8	0.0	3.8	0.3	10.0	0.0	5.0	0.2	5.0	0.5	5.0	1.4	25	0.8	15.0			0.4	5
P. fellutanum Biourge	3.0	23.1	4.1	15.4	4.7	15.0	2.4	20.0	3.8	25.0	0.9	5.0	0.4	5	0.5	5.0	0.2	5		
P. funiculosum Thom	6.6	61.5	1.8	61.5	5.8	40.0	11.8	60.0	7.1	35.0	11.9	60.0	5.8	45.0	9.7	40.0	8.3	40	10.8	55
P. islandicum Sopp	3.6	3.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. mirabile Beliakova & Milko	0.1	3.8	-	-	0.1	10.0	-	-	-	-	-	-	-	-	-	-	0.1	5	-	-
P. oxalicum Currie & Thom	0.1	3.8	0.5	30.8	0.4	15.0	0.1	5.0	-	-	2.6	20.0	0.1	5.0	0.8	15.0	5.5	20	1.3	5

P. pinophilum Hedgcock	-	-	0.8	23.1	0.1	5.0			1.3	15.0	0.6	10.0	1.3	15.0	2.6	30.0	-	-	0.4	10
P. purpurgenum Stoll	0.1	3.8	0.1	7.7	0.2	10.0	0.1	5.0	1.2	5.0	2.0	10.0	0.3	10.0	0.3	5.0	-	-	-	-
P. waksmanii Zaleski	0.1	3.8	0.0	3.8	-	-	-	-	3.1	15.0	2.0	15.0	0.1	5.0	-	-	3.9	20	-	-
Phoma Saccardo	0.3	14.5	0.4	19.2	-	-	-	-	-	-	-	-	-	-	-	-	1.4	10	-	-
P. exigua Desmazieres			0.1	3.8	-	-	-	-	-	-	-	-	-	-	-	-			-	-
P. herbarum Westendorp	0.3	14.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. leveillei Boerema & Bollen	-	-	0.3	19.2	0.1	10.0	0.1	5.0	2.0	5.0	-	-	-	-	-	-	1.4	10	-	-
Rhizoctonia solani Kühn	-	-			0.1	15.0			1.3	15.0	-	-	-	-	-	-	2.1	10	-	-
Rhizopus stolonifer (Ehrenberg) Vuillemin	6.6	84.6	6.4	65.4	6.3	65.0	6.8	65.0	14.5	70.0	10.4	60.0	3.6	25.0	4.0	25.0	0.4	5	10.6	35
Setosphaeria rostrata Leonard	-	-	0.2	11.5	0.1	5.0	-	-	-	-	0.6	10.0	-	-	-	-	-	-	-	-
Sordaria fimicola (Roberge) Cesati & de Notaris	6.3	3.8	0.1	3.8	-	-	-	-	-	-	0.1	5.0	-	-	-	-	-	-	-	-
Stachybotrys Corda	0.2	15.4	0.2	15.4	0.1	10.0	0.2	15.0	-	-	-	-	0.4	15.0	-	-	-	-	-	-
S. chartarum (Ehrenberg) Hughes	-	-	0.1	7.7			0.1	10.0	-	-	-	-	0.4	15.0	-	-	-	-	-	-
S. elegans (Pidopl.) W. Gams	0.2	15.4	0.1	7.7	0.1	10.0	0.1	5.0	-	-	-	-			-	-	-	-	-	-
Stemphylium Wallroth	0.9	19.2	0.2	15.4	0.3	5.0	-	-	-	-	-	-	0.9	25.0	2.6	35.0	3.9	15	3.9	15
S. botryosum Wallroth	0.5	15.4	0.1	7.7	0.3	5.0	-	-	-	-	-	-	0.7	20.0	1.7	30.0	3.5	15	3.3	10
S. vesicarium (Wallr.) E.G. Simmons	0.4	3.8	0.1	7.7	-	-	-	-	-	-	-	-	0.1	5.0	0.9	10.0	0.5	5	0.6	5.
Sterile mycelia	-	-	0.1	11.5	-	-	-	-	-	-	-	-	0.1	5.0	-	-	-	-	-	-
Trichoderma Persoon	3.9	30.8	3.0	38.5	0.8	20.0	2.3	25.0	5.6	40.0	3.0	10.0	10.0	45.0	5.9	45.0	5.9	20	2.4	10
T. hamatum (Bonorden) Bainier	1.5	3.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T. harzianum Rifai	0.8	19.2	1.9	30.8	0.2	5.0	1.0	10.0	5.6	40.0	3.0	10.0	8.4	35.0	3.2	30.0	5.9	20	2.4	10
T. koningii Oudem	0.9	3.8	1.1	3.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T. longibrachiatum Rifai	0.7	3.8	0.0	3.8	0.7	20.0	1.2	15.0	-	-	-	-	1.6	15.0	2.7	20.0	-	-	-	-
Zopfiella latipes (N. Lundq.) Malloch & Cain	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	10
Total counts	1863876	552360			679945		572980		1276		1327		133500		131300		811		944	
No. of genera 32	25	26			23.0		19.0		16.0		13.0		15		14		14		12	
Number of species 79	58	60			47.0		40.0		30.0		28.0		37		36		28		25	

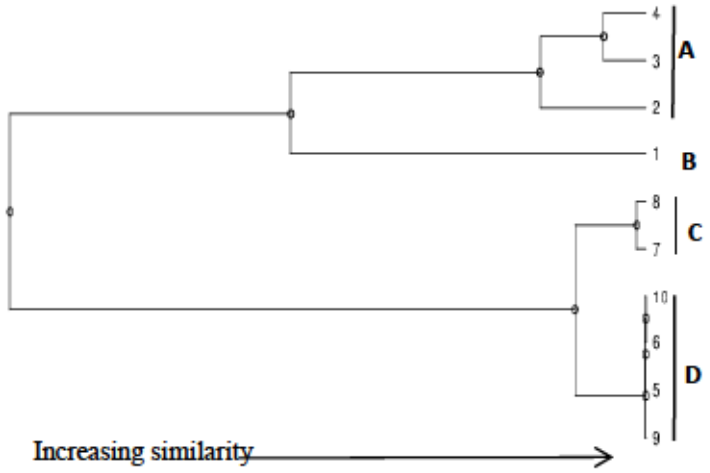

Figure 1 Cluster analysis of 5 sources; soil at 19°C incubation temperature (1), soil at 28°C (2), rhizosphere at 19°C (3), rhizosphere at 28°C (4), rhizoplane at 19°C (5), rhizoplane at 28°C (6), phyllosphere at 19°C (7), phyllosphere at 28°C (8), phylloplane at 19°C (9) and phylloplane at 28°C (10) based on the similarity of their fungal communities using species total counts. The sources cluster into four major groups (A to D). Sources of group C are similar to those of group D, while groups A and B have low similarity with groups C and D.

New record species in Egypt

A fungal species was recorded in this study from phylloplane (green leaves of onion plant at 28°C) for the first time in Egypt, namely *Zopfiella latipes* . It was isolated from samples and accounting 0.32% of total phylloplane fungi (Table).
***Zopfiella latipes* (Lundqvist) Malloch and Cain 1971**
Synonym: *Tripterospora latipes* **Lundqvist, 1969**

Macroscopic features: On PDA and glucose-Czapek's agar, colony grows fast, attaining 9 cm in diameter after 10 days at 28°C, greyish brown to olive brown, velutinous; reverse dark brown.

Microscopic features: Ascomata pale greyish brown or dark brown to black, globose or subglobose, superficial, non-ostiolate, irregularly dehiscing, 120-205 µm in diameter, and covered with hyphae. Peridium semitransparent, with soft skin, composed of three or four layers of irregular or angular thin-walled pseudoparanchymatous cells (Figure 4 A,B). Asci are 8-ascosporous, thin walled, clavate, broadest in the middle, 80-120×14-18 µm, apically truncate (Figure 4C). Ascospores biseriate, ellipsoidal, globulate, becoming one-septate in the lower third, slightly constricted at the septum, 2-celled; large upper cell 16-25 x 12-15 µm, ellipsoidal, apex conical or umbonate, base truncate, olivaceous to brown, thin-walled, smooth, with a subapical germ pore, 1 µm in diameter, lower cell small, 4-8 x 3.5-7µm, broadly cylindrical, apex truncate, base broadly rounded, hyaline **(Malloch and Cain, 1971; Furuya and Udagawa, 1973)** (Figure 4B-D). *Zopfiella latipes* belongs to Phylum: Ascomycota, Subdivision: Pezizomycotina, Class: Sordariomycetes, Subclass: Sordariomycetidae, Order: Sordariales, Family: Lasiosphaeriaceae.
It was isolated from soil and wood immersed in sea water **(Malloch and Cain, 1971)**, from freshwater habitats in Florida Peninsula **(Raja *et al.*, 2009)**, from three mangrove plants in India **(Manimohan *et al.*, 2011)**.

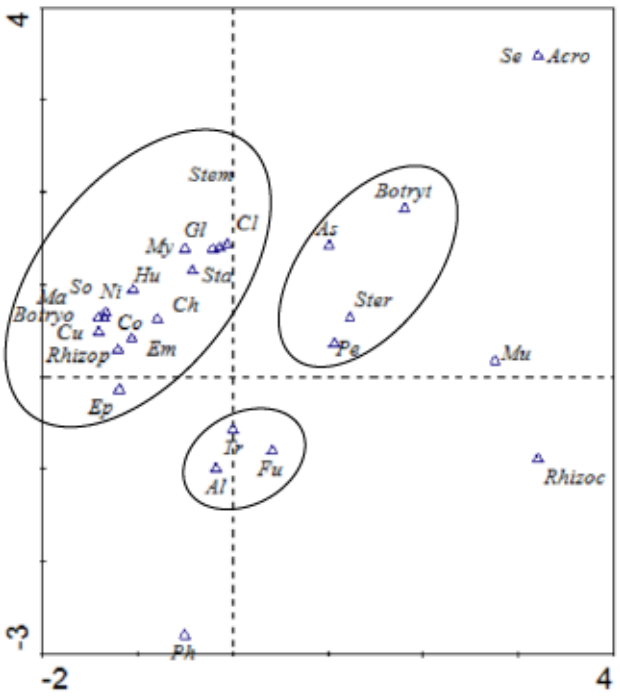

Figure 3a (left). Testing of differences in fungal genera composition of soil, roots and green leaves of onion isolated at 19ºC; DCA ordination diagram showing the position of fungal genera in different sources; *Acrophalophora* (Acro), *Alternaria* (Al), *Aspergillus* (As), *Botryotrichum* (Botryo), *Botrytis* (Botryt), *Chaetomium* (Ch), *Cladosporium* (Cl), *Cochliobolus* (Co), *Cunninghamella* (Cu), *Emericella* (Em), *Epicoccum* (Ep), *Fusarium* (Fu), *Gliocladium* (Gl), *Humicola* (Hu), *Macrophomina* (Ma), *Mucor* (Mu), *Nigrospora* (Ni), *Penicillium* (Pe), *Phoma* (Ph), *Rhizoctonia* (Rhizoc), *Rhizopus* (Rhizop), *Setosphaeria* (Se), *Sordaria* (So), *Stachybotrys* (St), *Stemphylium* (Stem), Sterile mycelia (Ster) and *Trichoderma* (Tr).

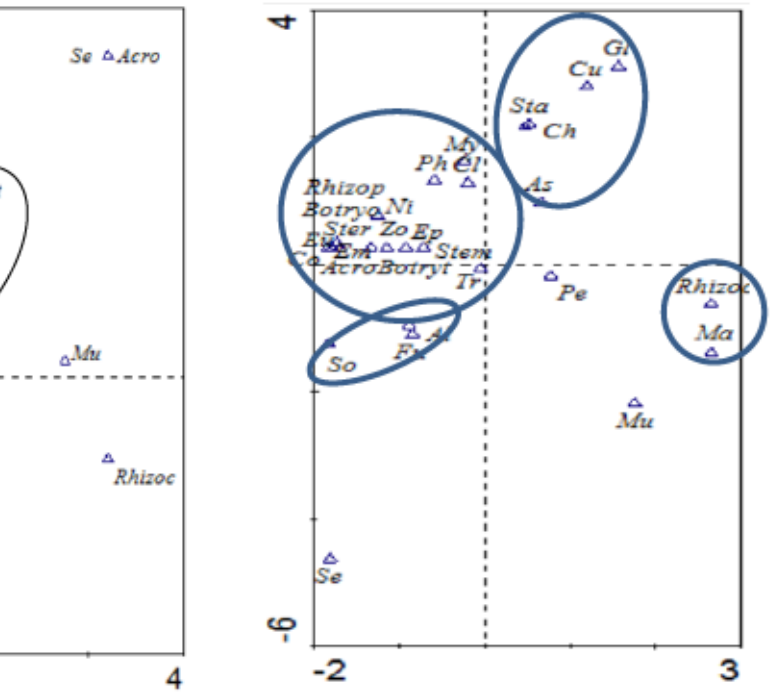

Figure 3b (right). Testing of differences in fungal genera composition of soil, roots and green leaves of onion isolated at 28ºC; DCA ordination diagram showing the position of fungal genera in different sources; *Acrophalophora* (Acro), *Alternaria* (Al), *Aspergillus* (As), *Botryotrichum* (Botryo), *Botrytis* (Botryt), *Chaetomium* (Ch), *Cladosporium* (Cl), *Cochliobolus* (Co), *Cunninghamella* (Cu), *Emericella* (Em), *Epicoccum* (Ep), *Eurotium* (Eu), *Fusarium* (Fu), *Gliocladium* (Gl), *Macrophomina* (Ma), *Mucor* (Mu), *Mycothecium* (My), *Penicillium* (Pe), *Phoma* (Ph), *Rhizoctonia* (Rhizoc), *Rhizopus* (Rhizop), *Setosphaeria* (Se), *Sordaria* (So), *Stachybotrys* (St), *Stemphylium* (Stem), Sterile mycelia (Ster) and *Trichoderma* (Tr).

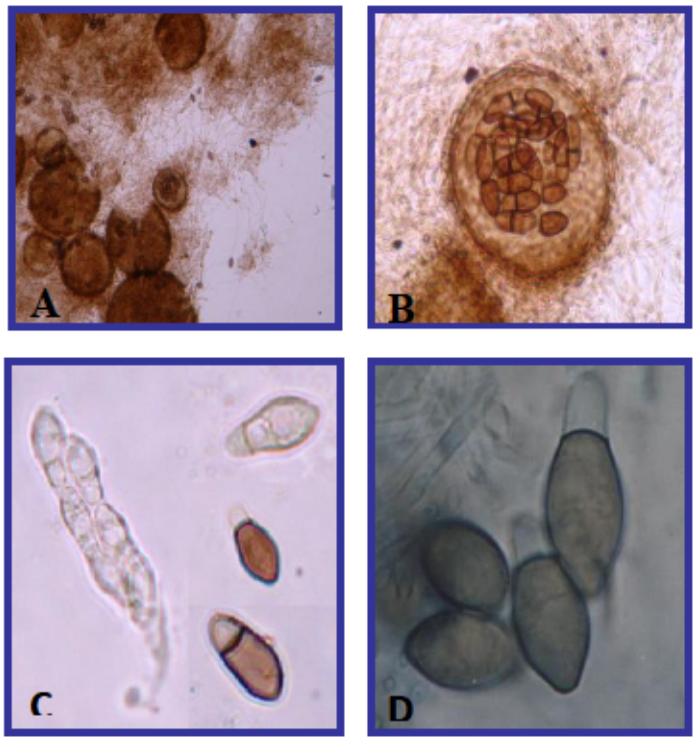

Figure 4 *Zopfiella latipes*: Non ostiolate ascoma (A and B); Clavate asci, with 8-ascosporous & mature and immature ascospores (C); 2-celled ascospores (D)

CONCLUSION

The fungal compositions of soil, root and leaf surfaces fungi associated with onion plant were determined, resulting in collecting 79 species belonging to 32 genera, of which *Zopfiella latipes* was recorded during this investigation for the first in Egypt. The fungal species isolated from soil at 28ºC and rhizosphere at 19º and 28ºC are similar to fungal community in soil at 19ºC. Also, fungal communities isolated from phyllosphere at both 19º and 28ºC are basically similar.

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ENZYMATIC CHARACTERIZATION OF YEAST STRAINS ORIGINATED FROM TRADITIONAL MIHALIC CHEESE

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ABSTRACT

Yeasts, associated with secondary flora of many cheese types, are important microorganisms for cheese ripening process. The aim of this study was to identify the yeasts isolated from traditional Mihalic cheese and to determine their enzymatic activities as a tool for their technological characteristics. Phenotypic identification was performed by using API ID 32C test system and some complementary morphological, physiological and biochemical tests. Enzyme profiles of the isolates were determined by using API-ZYM strips. In this study, 72 yeast isolates were obtained from 29 Mihalic cheese samples. Fifty-six (78%) of the isolates could be identified at species level, and one isolate at genus level. The identified yeast species belonged to three genera; *Candida, Geotrichum* and *Trichosporon*. It was determined that *Candida famata* var. *famata* was the dominant species in Mihalic cheese. The yeast isolates had variable enzyme activities including acid phosphatase, esterase, esterase lipase, lipase, β-galactosidase, leucine arylamidase, valine arylamidase and cysteine arylamidase, which could have important attributes during cheese ripening. *C. famata var. famata* M22, *Candida guilliermondii var. membranefaciens* M54 and *Candida tropicalis* M2 were selected to be superior strains on the basis of their enzyme profiles. Identification and enzymatic characterization of the yeasts originated from Mihalic cheese was performed for the first time in this study.

Keywords: Mihalic cheese, yeast, isolation, identification, enzymatic characterization

INTRODUCTION

Mihalic cheese is one of the traditional cheeses of Turkey, widely produced in the provinces of Bursa, Balıkesir and Canakkale (**Kamber, 2008**). The name "Mihalic" is an old name of Karacabey, county of Bursa, and it is known that the cheese has been produced in these areas for at least 250 years (Hayaloglu *et al*., 2008). Among the inhabitants of the region, the cheese is also known by such different names as Maglic, Mahlic, Kelle or Manyas cheese (**Aday & Karagul Yuceer, 2014**). It is assumed that Mihalic cheese was adopted from Greek cheese-making traditions (**Kamber, 2008**). It is a brined cheese made from raw sheep's or goat's milk and characterized by high levels of salt and dry matter (**Kamber, 2008; Aday & Karagul Yuceer, 2014**). It is ripened in wooden barrels at 15-25°C for three months in brine (**Aday & Karagul-Yuceer, 2014**). It is tough, tightly-structured and ranges from cream to light yellow in color. It has a characteristic sharp taste and odor and has 3-4 mm diameter pores gradually decreasing from the center to the sides in its cross-section (**Kamber, 2008**).

It is known that composition and activity of the microflora in cheese play a critical role in the formation of cheese, leading to production of diverse range of cheese varieties (**Jany & Barbier, 2008**). It was reported that the recovery of yeasts in high numbers from cheeses (e.g. 10^6–10^9 cfu/g) and their ability to hydrolyse milk fat and proteins have suggested that they influence the organoleptic characteristics of cheese. Even in cheeses inoculated with bacterial starters, yeasts are reported to be detected at counts as high as 10^8 cfu/g (**Capece & Romano, 2009**). Yeast species usually represent secondary microbiota in cheeses and among them *Kluyveromyces marxianus*, *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica* (**Golić *et al*., 2013**), *Kluyveromyces lactis* (**Gardini *et al*., 2006**), *Trichosporon cutaneum*, *Candida zeylanoides* and *Geotrichum candidum* (**Padilla *et al*., 2014a**) were reported to be dominant species in various cheeses. It has been reported that the yeasts play an important role in proteolysis, lipolysis, fermentation of residual lactose, and assimilation of lactic and citric acid during the ripening of cheese, contributing to aroma development and to the rheological properties of the final product (**Gardini *et al*., 2006; Padilla *et al*., 2014b**) . Additionally, some cheese yeasts have been recognized by their probiotic character and DNA-bioprotective action against model genotoxins (**Padilla *et al*., 2014b**).

With regard to traditional cheeses in Turkey, with exception of a few varieties, most of the traditional brined cheeses have not yet been industrialized. It has been reported that relatively little is known about the basic and microbiological characteristics of the brined cheeses native to Turkey (**Hayaloglu *et al*., 2008**). There are a few reports about the chemical and microbiological properties of Mihalic cheese (**Solak & Akin, 2013; Aday & Karagul Yuceer, 2014**). The reports concerning microflora of Mihalic cheese were usually focused on pathogenic bacteria (**Cokal *et al*., 2012**). To date, there is no reported study about the yeast flora of Mihalic cheese. The aim of this study was to determine the predominant yeast species in the microflora of Mihalic cheese and to characterize their enzymatic activities.

MATERIALS AND METHODS

Cheese samples and chemical analysis

Thirty-one samples of Mihalic cheese were randomly collected from the markets in Ankara and transported to the laboratory. Most of the cheese samples were packaged in polyethylene bags, and the others were aseptically taken from the newly opened tin boxes in the markets. Water activity, pH, dry solid content and salt content of all samples were analysed in duplicate. Water activity was measured based on "dew point" method (**Fontana, 1998**) by using a water activity measurement device (Aqualab Model CX2, Decagon, USA). Dry solid content was determined according to **Bradley (1998)** and salt content was determined by Mohr method described by **Hendricks (1998)**.

Isolation of yeasts

Cheese samples were cut into small pieces under aseptic conditions and 25 grams from each block (sample) were homogenised with 225 mL of 0.1% (w/v) peptone water in a stomacher (Seward Stomacher 400 Type BA 7021, UK). For isolation of the yeasts, appropriate dilutions were inoculated on Yeast Extract Dextrose Chloramphenicol (YDC) agar (Lab M, UK) and Dichloran 18% Glycerol (DG18) agar plates (**Deák & Beuchat, 1996; Pitt & Hocking, 1997**). After incubation at 28°C for 2-7 days, the colonies grown on both media were randomly selected on

the basis of their macroscobic morphology. Colonies with different morphology were inoculated into Yeast Extract Malt Extract (YM) agar (Lab M, UK) and pure cultures were obtained at 28ºC for 48 hours. The pure cultures were maintained at 4°C, until use.

Identification of the yeast isolates

Yeast isolates were identified by using API ID 32C, a rapid miniaturised identification system (bioMérieux, France), and some complementary tests. API ID 32C strips were used according to the suppliers instructions. The results of API ID 32C was evaluated by using Apilab Plus, a specific computer programme developed for API ID 32C strips and mini API analyser (bioMérieux, France). The complementary identification tests used were as follows; macroscobic and microscobic morphologies (**Pitt & Hocking, 1997; Kurtzman *et al.*, 2003**), growth characteristics in liquid medium (**Kurtzman *et al.*, 2003**), glucose fermentation (**Harrigan, 1998; Yarrow, 2000; Kurtzman *et al.*, 2003**), urea hydrolysis (**Deák & Beuchat, 1996; Yarrow, 2000; Kurtzman *et al.*, 2003**), nitrate assimilation (**Deák & Beuchat, 1996; Yarrow, 2000**) growth at 50% and 60% glucose concentrations (**Yarrow, 2000; Kurtzman *et al.*, 2003**), growth at 37ºC, growth in media including 0.5% and 1% acetic acid (**Pitt & Hocking, 1997**), pseudohyphae and ascospore formations (**Yarrow, 2000**). For some of the isolates; galactose fermentation, growth in medium without vitamin (**Yarrow, 2000**) and growth in medium including 16% NaCl and 5% glucose were also investigated.

Determination of enzymatic activities

Enzymatic activities of the yeast isolates were screened by using miniaturized API-ZYM test system (bioMérieux, France), which enables screening 19 different enzyme activities. After activation of the yeast cultures on YM agar at 30ºC for 24 hours, they were suspended in distilled water until suspensions reached 5 or 6 McFarland turbidity. The suspensions were inoculated in the microwells on the API-ZYM strip at a level of 65 µL for each cupule. After incubation at 37ºC for 4 - 4.5 hours, ZYM A and ZYM B reagents were added to each cupule and after five minutes, strips were put under 1000 W lamp for 10 seconds for prevention of yellow colour formation caused by Fast Blue BB. For evaluation of the results, each enzyme activity was graded from 0 to 5 by comparing developed colour with the API-ZYM colour reaction chart. The approximate amount of free hydrolysed substrate (nmol) could be obtained from the colour strength: 0, no activity; 1, liberation of 5 nmol; 2, 10 nmol; 3, 20 nmol; 4, 30 nmol; 5, ≥ 40 nmol. The grades were evaluated as no activity (0), low activity (1), intermediate activity (2-3) and high activity (4-5).

For screening enzymatic activities, 30 of the 56 identified isolates were used. Thirty isolates were selected according to their biochemical and physiological characteristics. All strains belonging to the same species with different biochemical and physiological characteristics were enzymatically characterized.

RESULTS

Results of chemical analysis

The analysed thirty-one Mihalic cheese samples differed in some chemical properties. Water activities and pH values of the samples were found to change between 0.785±0.001-0.954±0.001 and 3.62±0.08 - 4.58±0.10, respectively. Dry solid content of the Mihalic cheese samples were in the range of 55.76±0.12 - 71.67±0.18 (%), while salt content on dry basis was determined to change between 3.46±0.01-13.99±1.63 (%) (data not shown).

Results of yeast isolation and identification

Isolation experiments resulted in obtaining 72 yeast isolates from 29 of the 31 cheese samples. The isolates were coded with "M" and numbers. According to the identification results obtained with API ID 32C, 43 of the isolates could be identified at species level. In species identification, an identification category of "excellent" as established by the manufacturer was obtained for 4 of the isolates. The identification profile was defined as "very good" and "good" for 24 and 15 of the isolates, respectively. Eleven isolates could be identified at genus level as "*Candida*", 2 isolates as "*Geotrichum*". Identification categories of 11 of the isolates were defined as "unacceptable profile", one of the isolates as "acceptable profile", two of them as "doubtful" profile, one of them as "low discrimination", and one of them gave no identification result with the use of API ID 32C strips (data not shown). As a result, ID 32C strips did not give satisfactory identification results for 16 of the isolates. According to the identification results obtained with API ID 32C, species identification levels of the yeasts were changed between 96.6-99.9%. Identification at genus level was achieved between the range of 67.5-94.4% (data not shown). The identified isolates were in the species of; *Trichosporon asahii* (1), *Candida tropicalis* (3), *Candida insconspicua/norvegensis* (3), *Candida famata* (29), *Saccharomyces cerevisiae* (1), *Candida catenulata* (1), *Kodamaea ohmeri* (1), *Candida krusei* (2) and *Candida zeylanoides* (2).

The complementary identification tests were used for the isolates which could not be identified, or identified only at genus level with API ID 32C. The results of these tests were represented in Table 1.

Table 1 Results of some complementary tests used for the identification of the isolates

Tests / Yeast species	*C. bertae*	*C. catenulata*	*C. cylindracea*	*C. famata* var *famata*	*C. guilliermondii* var *membranefaciens*	*C. krusei*	*C. norvegensis*	*C. paludigena*	*C. robusta*	*C. tropicalis*	*C. zeylanoides*	*G. candidum*	*T. asahii*
Glucose fermentation	-	+	v	v	+	+	+	-	+	+	v	-	-
Urea hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	+
Nitrate assimilation	+	-	-	-	-	-	-	-	-	+	-	-	-
Growth at 50% glucose	+	+	v	+	+	-	-	+	+	-	-	-	-
Growth at 60% glucose	v	-	-	v	+	-	-	v	-	-	-	-	-
Growth at 37°C	-	-	-	-	-	v	v	-	-	+	-	v	+
Growth at 0.5% acetic acid	-	-	-	-	-	v	-	-	-	-	-	-	-
Growth at 1% acetic acid	-	-	-	-	-	-	-	-	-	-	-	-	-
Ascospore formation	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudohyphae formation	-	+	v	v	+	+	v	v	-	+	v	+	-
Growth on media without vitamin[1]	*	*	*	*	*	*	*	*	*	*	*	-	*
Galactose fermentation[1]	*	*	+	*	*	*	*	*	*	*	*	*	*

(+): Positive. (-): Negative. (v): Variable. ([1]): These tests were used for only some of the isolates. (*): Not used for these isolates

When the assimilation test results of API ID 32C system and complementary identification tests were evaluated together by using identification keys of **Barnett *et al.* (2000)**, **Payne *et al.* (2000)** and **Kurtzman & Fell (2000)**, identification at species level was achieved for some of the isolates. By this way, some of the isolates which could not be identified or identified at genus level could be identified at species level. For example, among the isolates which were identified at genus level as "*Candida*", the strains M53 and M63 could be identified as *Candida bertae* by using identification keys of **Payne *et al.* (2000)**. By the same way, four isolates (M32, M48, M79, M88) could be identified as *Candida paludigena*, while the other four (M73, M77, M78, M83) were identified as *Candida cylindraceae*. For identifying *C. cylindraceae* isolates, galactose fermentation test was used in addition to other complementary tests as recommended by **Payne *et al.* (2000)**. For the strains M3, M52 and M56, identification result of API ID 32C strips were given as *C. insconspicua* or *C. norvegensis*. They were identified as *C. norvegensis* because of their ability to ferment glucose, as described by **Meyer *et al.* (2000)** and **Barnett *et al.* (2000)**. Thirty isolates which were identified as *C. famata*, were in the variety of *C. famata* var. *famata* according to **Meyer *et al.* (2000)**, since they could not grow at 37ºC. The profile of the isolates M6 and M15 were defined as *Geotrichum* spp. by the API ID 32C strips. They were further identified as *G. candidum* by using

the test of growth on the media without vitamin as recommended in the identification keys of **Payne *et al.* (2000)**. By using the ascospor formation tests, the isolate M54 which was identified as *K. ohmeri* by the strips, was determined to be its anamorph *Candida guilliermondii* var. *membranaefaciens*. Similarly, the isolate M8 was identified as *Candida robusta*, the anamorph form of *S. cerevisiae*.

The final identification results were given in Table 2. By using complementary tests in addition to API ID 32C strips, 56 (78%) of the isolates could be identified at species level, and one isolate at genus level as "*Candida*". It was determined that *C. famata* var. *famata* (30) was the dominant species in Mihalic cheese. *C. cylindracea* (4), *C. paludigena* (4), *C. tropicalis* (3), *C. norvegensis* (3), *C. krusei* (2), *C. zeylanoides* (2), *G. candidum* (2), *C. bertae* (2), *C. catenulata* (1), *C. guilliermondii* var. *membranaefaciens* (1), *C. robusta* (1) and *T. asahii* (1) were also among the detected species in the microflora of Mihalic cheese.

Table 2 The species and number of identified yeast isolates

Yeast species	Number of isolates
Candida bertae	2
Candida catenulata	1
Candida cylindracea	4
Candida famata var *famata*	30
Candida guilliermondii var *membranefaciens*	1
Candida krusei	2
Candida norvegensis	3
Candida paludigena	4
Candida robusta	1
Candida tropicalis	3
Candida zeylanoides	2
Candida sp.	1
Geotrichum candidum	2
Trichosporon asahii	1
Not identified	15

Results of enzymatic characterization

The results obtained by API-ZYM strips were given in Table 3. It was found that none of the isolates had α-galactosidase, β-glucuronidase, N-acetyl-β-glucoaminidase, α-mannosidase and α-fucosidase activities. Fifty-percent of the strains had β-galactosidase activity. The highest activity of this enzyme was observed for *C. paludigena* M32. Only two strains; *Trich. asahii* M1 and *C. tropicalis* M2 had β-glucosidase activity. Seventeen (55%) of the isolates showed α-glucosidase activity. Alkaline phosphatase activity was detected for most of the isolates and all of the *C. famata* var. *famata* strains. All of the isolates except two *C. norvegensis* strains, showed acid phosphatase activity. The level of acid phosphatase activity was high (5) for *Trich. asahii* M1, *C. tropicalis* M2, *C. krusei* M57, *C. guilliermondii* var. *membranefaciens* M54, *G. candidum* M15 and *C. zeylanoides* M16 (Table 3). All of the isolates had esterase (C4) and esterase lipase (C8) activities. However, only *C. tropicalis* M2, *C. famata* var. *famata* M22 and *G. candidum* M15 were found to have lipase (C14) activity.

The enzyme leucine arylamidase was detected in all of the isolates, while valine and cysteine arylamidases were found only in a few isolates at very low levels. Among proteases, trypsin and chymotrypsin, which operate most effectively in alcaline pH values, were not detected in most of the isolates of Mihalic cheese, an acidic product. Figure 1 represents the percentage of the yeast strains with certain enzyme activities detected by API-ZYM. The enzymes esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphtol-AS-BI-phosphohydrolise and also alkaline phosphatase were common among the isolates of Mihalic cheese (Table 3).

Table 3 Enzyme activities of the yeast isolates

Isolate no.	Yeast species	Control	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
M1	*T. asahii*	0	3	2	2	0	3	0	0	0	0	5	5	0	0	0	0	2	0	0	0
M2	*C. tropicalis*	0	0	1	2	1	4	1	1	0	0	5	2	0	0	0	4	2	0	0	0
M43	*C. tropicalis*	0	0	2	1	0	3	1	0	0	0	4	2	0	0	0	3	0	0	0	0
M25	*C. catenulata*	0	2	3	2	0	4	1	1	0	0	2	2	0	0	0	0	0	0	0	0
M3	*C. norvegensis*	0	0	1	2	0	3	0	2	0	0	0	1	0	0	0	0	0	0	0	0
M56	*C. norvegensis*	0	0	2	2	0	3	1	2	0	0	0	2	0	0	0	0	0	0	0	0
M57	*C. krusei*	0	0	1	2	0	3	1	2	0	0	5	3	0	0	0	0	0	0	0	0
M54	*C. guilliermondii* var. *membranefaciens*	0	1	2	1	0	5	0	0	0	0	5	1	0	0	0	3	0	0	0	0
M18	*C. famata* var. *famata*	0	3	3	2	0	2	0	1	0	1	3	2	0	2	0	2	0	0	0	0
M91	*C. famata* var. *famata*	0	2	2	2	0	2	0	0	0	0	2	4	0	3	0	1	0	0	0	0
M21	*C. famata* var. *famata*	0	2	2	1	0	3	0	0	0	0	3	1	0	2	0	1	0	0	0	0
M89	*C. famata* var. *famata*	0	2	2	2	0	3	0	1	0	0	3	3	0	1	0	1	0	0	0	0
M22	*C. famata* var. *famata*	0	3	3	2	1	3	1	1	1	1	3	2	0	2	0	2	0	0	0	0
M70	*C. famata* var. *famata*	0	3	2	1	0	2	0	1	0	0	3	1	0	2	0	1	0	0	0	0
M45	*C. famata* var. *famata*	0	1	2	1	0	2	0	0	0	0	1	1	0	3	0	2	0	0	0	0
M12	*C. famata* var. *famata*	0	2	2	1	0	2	0	0	0	0	3	2	0	2	0	1	0	0	0	0
M81	*C. famata* var. *famata*	0	3	1	2	0	2	1	0	0	0	4	2	0	0	0	0	0	0	0	0
M4	*C. famata* var. *famata*	0	1	2	1	0	2	0	0	0	0	1	1	0	2	0	1	0	0	0	0
M6	*G. candidum*	0	0	1	2	0	3	1	1	0	0	4	1	0	0	0	0	0	0	0	0
M15	*G. candidum*	0	1	2	3	2	1	1	0	1	0	5	1	0	0	0	0	0	0	0	0
M8	*C. robusta*	0	1	2	1	0	2	1	0	0	0	2	1	0	3	0	2	0	0	0	0
M16	*C. zeylanoides*	0	0	2	2	0	2	0	0	0	0	5	1	0	0	0	0	0	0	0	0
M76	*C. zeylanoides*	0	0	1	2	0	1	0	0	0	0	3	2	0	0	0	0	0	0	0	0
M73	*C. cylindracea*	0	1	3	2	0	4	1	1	0	0	1	1	0	0	0	0	0	0	0	0
M77	*C. cylindracea*	0	2	2	2	0	5	1	1	0	0	2	2	0	0	0	0	0	0	0	0
M83	*C. cylindracea*	0	2	2	1	0	1	0	0	0	0	3	1	0	3	0	1	0	0	0	0
M79	*C. paludigena*	0	1	2	1	0	2	0	0	0	0	2	2	0	3	0	0	0	0	0	0
M32	*C. paludigena*	0	2	2	1	0	2	0	0	0	0	3	1	0	4	0	1	0	0	0	0
M63	*C. bertae*	0	1	2	1	0	2	0	0	0	0	3	1	0	3	0	1	0	0	0	0
M53	*C. bertae*	0	3	3	2	0	2	0	0	0	0	3	1	0	3	0	1	0	0	0	0

1: Alkaline phosphatase, 2: Esterase (C4), 3: Esterase lipase (C8), 4: Lipase (C14), 5: Leucine arylamidase, 6: Valine arylamidase, 7: Cystine arylamidase, 8: Trypsin, 9: α-chymotrypsin, 10: Acid phosphatase, 11: Naphtol-AS-BI-phosphohydrolise, 12: α-galactosidase, 13: β- galactosidase, 14: β-glucuronidase, 15: α-glucosidase, 16: β-glucosidase, 17: N-acetyl- β-glucoaminidase, 18: α-mannosidase, 19: α-fucosidase

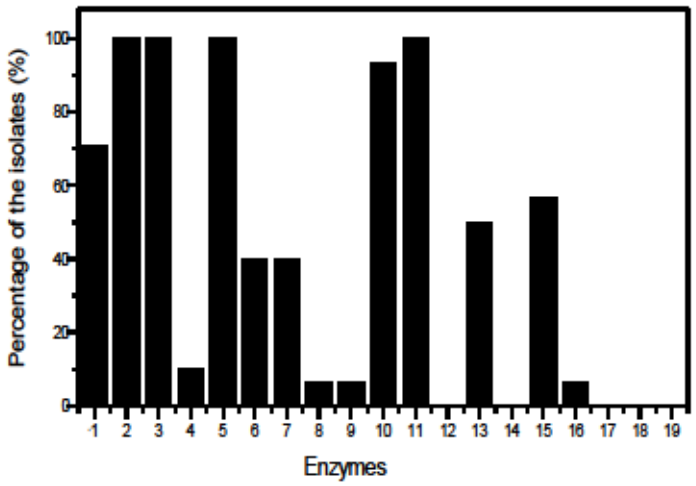

Figure 1 Percentage of the yeast isolates (%) having the certain enzyme activity
1: Alkaline phosphatase, 2: Esterase (C4), 3: Esterase lipase (C8), 4: Lipase (C14), 5: Leucine arylamidase, 6: Valine arylamidase, 7: Cystine arylamidase, 8: Trypsin, 9: α-chymotrypsin, 10: Acid phosphatase, 11: Naphtol-AS-BI-phosphohydrolise, 12: α-galactosidase, 13: β- galactosidase, 14: β-glucuronidase, 15: α-glucosidase, 16: β-glucosidase, 17: N-acetyl- β-glucoaminidase, 18: α-mannosidase, 19: α-fucosidase

DISCUSSION

C. famata var. *famata* was determined as the dominant yeast in Mihalic cheese. Although the prevalence of different yeast species depends on the type of the cheese considered, *D. hansenii* (anamorph: *C. famata*) was reported to be the most common species found in almost all types of cheeses, especially in most Mediterranian ewes' and goats' cheeses. This was attributed to its ability to grow in the presence of salt at low temperature and to metabolize lactic and citric acids (**Capece & Romano, 2009; Padilla *et al.*, 2014b**). *D. hansenii* was also an important component in the microflora of young white pickled cheeses of Serbia (**Golić *et al.*, 2013**), Pecorino di Filiano cheese (**Capece & Romano, 2009**), Pecorino Crotonese cheese (**Gardini *et al.*, 2006**), Spanish blue-veined Cabrales cheese (**Alvarez-Martin *et al.*, 2007**), Gorgonzola-style and Danish-style blue-veined cheeses (**Viljoen *et al.*, 2003**) and Danish surface-ripened cheeses (**Petersen *et al.*, 2002**). *D. hansenii* was defined as a "salt loving" yeast by **Prista & Louerio-Dias (2007)** and this characteristic of the yeast was explained by the capability of membrane potassium carriers to transport potassium into the cells, even in the presence of high concentrations of sodium. It was not unexpected for *D. hansenii* to dominate in the microflora of Mihalic cheese, which is known as a salty local cheese in Turkey.

API-ZYM system was used for detecting general enzyme profiles of the yeast strains. The particular enzymes which are important for cheese ripening are β-galactosidase, α-glucosidase, β-glucosidase, acid and alkali phosphatases, esterase, lipase, trypsin, α-chymotrypsin and arylamidases. According to the enzymatic profiles of the yeasts, the presence of β-galactosidase, α-glucosidase and β-glucosidase suggests that the yeast strains isolated from Mihalic cheese prefer glucose and lactose other than mannose, fructose and glucuronides as carbon and energy sources (**Heperkan *et al.*, 2014; Zeng *et al.*, 2014**). Acid and alkali phosphatases are given among the important enzymes for cheese ripening. It is known that although both acid and alkaline phosphatases are present in cheese, acid phosphatases are more active due to their relatively low optimum pH (**Magboul & McSweeney, 1999**). Acid phosphatase has a greater thermal stability and it is most active at pH values typical of cheese ripening (**Chavarri *et al.*, 1998**). It has been suggested that phosphatase activity could influence cheese flavor because of its effect on proteolysis. Acid phosphatase, acting synergistically with proteolytic enzymes, had been shown to hydrolyze casein molecule (**Akuzawa & Fox, 2004**). Phosphopeptides in milk have been reported to be resistant to proteolytic attack, and the combined action of acid phosphatases and proteolytic enzymes in cheese was thus required for extensive production of small peptides and free aminoacids (**Chavarri *et al.*, 1998; Akuzawa & Fox, 2004**). It was reported that the high acid phosphatase activity of microorganisms might be useful in metabolizing phosphates and contributing to flavor formation in acidic external environment prevalent in cheese maturation (**Akuzawa & Fox, 2004; Georgieva *et al.*, 2009**).

The strains having esterase and lipase activities are known to have potential to involve in the liberation of free fatty acids during cheese ripening (**Zeng *et al.*, 2014**). It was estimated that yeast strains having high esterase, esterase lipase or lipase activities might contribute to lipolysis in cheese ripening. Lipolysis plays an important role in cheese ripening, and a large number of studies dealing with the acceleration of lipolysis have been published (**Kheadr *et al.*, 2002; El Galiou *et al.*, 2013**). The free fatty acids released during lipolysis contribute, together with the volatile compounds and the proteolysis products, directly to cheese flavor (**El Galiou *et al.*, 2013**). The presence of microorganisms with high aminopeptidase activity was reported as advantageous for cheese ripening and flavor development (**Georgieva *et al.*, 2009**). Arylamidases (aminopeptidases) catalyze the hydrolysis of N-terminal aminoacids from peptide, amide or arylamides (**Dodor & Tabatabai, 2007**). According to **Zeng *et al.* (2014)**, aldehydes, alcohols and acids, which have very low treshold values, usually originate from the degradation of leucine, valine, phenylalanine and methionine. Aminopeptidases were also reported to have a debittering effect during cheese ripening (**Herreros *et al.*, 2003**). It was also reported that flavor development during ripening favor the detection of yeast strains with aminopeptidase activity (**Zeng *et al.*, 2014**). The absence of proteases (trypsin and chymotrypsin) and the high activities of peptidases and phosphatases among the strains tested were reported as desireable traits for flavor and texture development in milk fermentations (**Mathara *et al.*, 2004; Thapa *et al.*, 2006**). It was also reported that starters with low proteinase and strong peptidase activities were useful in reducing bitterness and improving body and texture defects (**Mathara *et al.*, 2004**). **Zeng *et al.* (2014)** reported that the enzymatic potential of microorganisms can effectively reproduce the characteristic flavour of fermented food rapidly, leading to more diverse flavours. Therefore, enzyme profiling can be used to select suitable strains as starter cultures.

When enzymatic activity results were evaluated in the present study, some yeast strains were found to stand out with their multiple enzyme activities. For selection of the superior strains, both enzyme activities and quantities should be taken into account. It is thought that the premier yeasts having particular enzymes important for cheese ripening could be subject of further investigations for their adjunct starter potential, which could be demonstrated by other technological properties of yeasts in addition to their enzymatic activities. The strain *C. famata* var. *famata* M22 had most of the enzymes (esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, □-chymotrypsin, acid phosphatase, □-galactosidase and □-glucosidase) which may have important attributes during cheese ripening. However, enzyme activities of this strain were generally low. Although low levels of proteases are recommended in some studies, other enzymes such as acid phosphatase and arylamidases may have more critical roles during ripening. Strains lack of proteases, but having high acid phosphatase and arylamidase activities could be also suggested to be potential adjunct starters for further studies. Thus, *C. guilliermondii* var. *membranefaciens* M54 with very high (level 5) acid phosphatase and leucine arylamidase activities could be also selected. Besides, *C. tropicalis* M2 may also be superior because of having all three arylamidases in addition to its high acid phosphatase activity.

CONCLUSION

This study demonstrated the yeast flora unique to Mihalic cheese which is one of the most important traditional cheeses of Turkey and also attracted attention in recent years as slow food. Identification and enzymatic characterization of the yeasts originated from Mihalic cheese was performed for the first time in this study. *C. famata* var. *famata* was determined as the dominant yeast. The yeast isolates had variable enzyme activities including acid phosphatase, esterase, esterase lipase, lipase, β-galactosidase, leucine arylamidase, valine arylamidase and cysteine arylamidase, which could have important attributes during cheese ripening. Enzymatic profiles of the yeast isolates revealed some of their technological properties. Effects of these strains on cheese quality as adjunct starters and change of their enzyme activities during ripening could be investigated in further studies.

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MITIGATION OF THE TOXIC EFFECTS OF XENOBIOTICS IN MUNG SEEDLINGS BY PLANT SYNERGISTIC BACTERIA

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ABSTRACT

In today's world, the problems due to the rising pollution are inevitable. A major contributor to this pollution is due to the effluents released by textile industries, chemical factories and others. They adversely affect the agricultural lands nearby leading to the accumulation of xenobiotics and heavy metals in the soil followed by barrenness. A great amount of effort had directed towards the exploration of remedial approach by various workers. Employing the indigenous microbes with degradative capabilities *in situ* had been a solution to it. Engineering of microbes is the latest trend for degrading a vast spectrum of compounds. The present study aims to isolate bacteria capable of degrading Phenol, Hexane, Toluene and Xylene to test their synergism with the test plant, *Vigna radiata*. We collected our soil sample from petroleum station. Three-gram negative bacteria (*Proteus* sp., *Pseudomonas* sp and *Aeromonas* sp) and two-gram positive bacteria (*Enterococcus* sp and *Streptococcus* sp) were isolated and identified with test compound degradative potential. They were further used to assess their synergism with the study plant by employing hydroponics technique at 100 ppm concentration of test compound. *Streptococcus*sp and *Proteus*sp gave a promising result followed by *Enterococcus* sp. The seedlings experienced a negative inhibitory effect with *Aeromonas* sp and *Pseudomonas* sp.

Keywords: Xenobiotics, Hydroponics, Synergism

INTRODUCTION

A country's major economic backbone is its natural resources, human resources and the agricultural empire it possesses. It was reported that over 70% of the rural homes depends on the agriculture as prime means of livelihood. Economically speaking this sector accounts for almost one-third of the country's GDP and is its single largest contributor (India Brand Equity Foundation). It makes it clear that anything that affects the mentioned sector would directly result in a major downfall of Indian economy **(Bhat, 2015).** Over the decades due to the increasing human intervention and for a race of development it has in many ways disrupted the ecological balance. Degradation in the quality of soil, low agricultural output and rapid spreading of the barren land belt is a reflection of this anthropogenic activities. Worldwide, soil is being seriously degraded as a result of increasing industrial, agricultural and civil activities. Soil contamination, both diffuse and localised, can lead to damage to several soil functions and contamination of surface- and groundwater **(Vamerali *et al.* 2010)**. Pali, a district of Rajasthan, is the largest erstwhile hand processing clusters, now gradually moving to power processing machines. The effluents discharged from these units cause environmental pollution. Textile effluents discharged from various textile processing units of Pali, flow about 55 kilometres downstream, making the groundwater in several riverbank villages unfit for drinking and irrigation and also cause an adverse effect on crops productivity and health of people residing in those areas **(Srivastava *et al.* 2014).** Pollution of soils by radionuclides may be of different kinds. An important type of radionuclides originates from the emission to the atmosphere, e.g. nuclear explosions (^{3}H) or reactor operations (^{85}Kr).The subsequent fallout of radionuclides with precipitation and infiltration causes pollution of different aqueous and terrestrial ecosystems **(Groudev *et al.* 2001).** The consumption of this radionuclides and heavy metal-contaminated food can seriously deplete some essential nutrients in the body that are further responsible for decreasing immunological defences, intrauterine growth retardation, impaired physio-social faculties, disabilities associated with malnutrition and high prevalence of upper gastrointestinal cancer rates **(Khan *et al.* 2008).** Researchers worldwide are finding ways to cope up with this problem, and a significant amount of work has been done. Phytoremediation, the use of plants for environmental restoration, is an emerging cleanup technology. It uses plants to reduce, remove, degrade or immobilise environmental toxins, primarily those of anthropogenic origin, with the aim of restoring area sites to a condition usable for private or public applications **(Mukhopadhyay and Maiti, 2010).** Research on natural plants is mainly focused on detecting heavy metal hyper-accu3mulator plants and the mechanism of absorption by analysing heavy metal content in the dominant plant **(Wang *et al.* 2004).** Oil spillage or oil pollution on soil has adverse effects on bodies of surface water used by drinking household, industrial purposes aquatic life and the vast tract of agricultural lands. **Emuh 2010** reported that mushroom inoculated in locally sourced substrates showed promise in bioremediation of crude oil polluted the soil. Current evidence suggests that in aquatic and terrestrial environments microorganisms are the chief agents for the biodegradation of molecules of environmental concern, including petroleum hydrocarbons. Hydrocarbon-degrading bacteria, yeast and fungi are widely distributed in marine, freshwater and soil habitats **(Balba *et al.* 1998).**
In this study, we aim to isolate those xenobiotic-degrading bacterial species which shows synergism with our test plant, *Vigna radiata*, so that the physiological ill effects observed due to stress induction by xenobiotic compounds in seedlings can reduce to a greater extent for better productivity. Up to the best of our knowledge, it is the first report on the use of bacteria to reduce the abiotic stress on *Vigna radiata* due to xenobiotic (Phenol, Toluene, Xylene and Hexane) stress.

MATERIAL AND METHODS

Collection and processing of sample

The soil sample collected from a petroleum station expecting to heavily contaminated with various hydrocarbons and xenobiotics. Soil suspension was prepared in saline and used for the screening of xenobiotic-degrading bacteria.

Enrichment of the bacteria

The enrichment of the bacteria was carried out by using Trypticase Soy Broth. The media was supplemented with crystal violet-phenol red to selectively enrich gram-negative bacteria and sodium azide was added to enrich gram-positive ones. Supplemented with 100 and 500 ppm of xenobiotic (phenol, toluene, xylene, and hexane) with respect to control.

Identification of the isolates

The isolates were identified through the biochemical test, and morphological characterization and the results were validated by Bergey's manual of determinative bacteriology.

Hydroponics analysis of the test plant

After two leaf stages of mungbean plants, culturing media were established which consisted 100ppm of xenobiotic supplementation (test group) along with biotic (bacteria+media+plant) and abiotic control (xenobiotic+media+plant) group respectively. The culture bottles were divided into four groups wherein each group 5different bacteria along with biotic control and abiotic control. The germinated seedlings were then transplanted in these culture bottles in the hydroponic system. The mouth of the bottles is wrapped with aluminium foil. The seedlings are placed on the foil by piercing it and inserting the root ensuring it touches the medium. All the culture bottles were then incubated at 26°± 2° C, 2000 lux illumination in tissue culture rack for two weeks. The root shoot lengths of the seedlings are noted, and the physical health of the seedlings is also periodically monitored.

RESULTS AND DISCUSSION

The present study aims to isolate and identify those bacteria possessing the potent degrading capacity of the xenobiotics (test compounds) *i.e.;* Phenol, Toluene, Xylene and Hexane. Bacteria possessing degradative capability may affect the physiological health of the plant and hence were tested for the synergism of the bacterial isolates with study plant through the hydroponic system.

A total of 20 isolates were obtained. Among them, nine were gram positive, and eleven were gram-negative bacteria based onGram staining. Morphological and biochemical characterization test was performed, and the isolates were identified by Bergey's manual of determinative bacteriology (Table 1). The isolates identified are *Proteus*sp., *Pseudomonas* sp.and*Aeromonas*sp. (gram negative) and *Enterococcus* sp and *Streptococcus* sp (gram positive) (Table 2). These isolates were tested for their ability to survive and degrade our test compounds at 100 ppm of xenobiotic concentration. *Pseudomonas* sp was found to tolerate and degrade xylene, phenol under *in vitro* conditions.A similar evidence of*Pseudomonas* degrading phenol and monochlorophenols from the soil samples adjacent to textile, pharmaceuticals, industries and automobile workshops has been reported **(Jame *et al.* 2010; Buitron and Gonzalez 1996; Razika 2010).** In another study, *Pseudomonas* was able to degrade toluene and xylene, but benzene was not metabolised **(Otenio *et al.* 2005; Worsey and Williams 1975; Hemalatha 2011; Nahar 2000).** *Pseudomonas* possess XYL, a Nonconjugative Xylene-Degradative Plasmid which helps it to degrade xylene **(Friello *et al.*, 1976).** *Aeromonas*sp. Degraded hexane, toluene and xylene. *Aeromonas*is capable of good growth on toluene in the range of 6.25 to 386 mg/l **(Nahar, 1999).***Proteus* sp was able to grow on hexane and toluene. It is also able to degrade Benzene, Toluene, Ethylbenzene and Xylene (BTEX) from heavily polluted sites. *Streptococcus* sp was able to grow on toluene, xylene and phenol. It is used in the degradation of phenol from oil contaminated soil **(Bhavna *et al.* 2010).** It is also used for the efficient biotransformation of phenol and its derivative by Catechol 2,3-Dioxygenase metabolism **(Mohite, 2010 and 2015).** *Enterococcus* sp was able to grow on hexane and xylene. It can degrade C.I. Reactive Red 195, an azodye that is extensively used in textile dyeing, paper, printing, colour photography, pharmaceutics, cosmetics and other industries **(Mate and Pathade 2012).**

The test of synergism between the isolates and the study plant was performed by Hydroponics. *Proteus*, *Enterococcus* and *Streptococcus* were being found to be more synergistic with the seedlings helped the plant improved the phenotype in the presence of the xenobiotic [Fig 1].The effect of the isolated bacteria on the root-shoot length of the seedlings was also evaluated [Fig 2].*Streptococcus* sp. was found to be performing maximum degradation followed by *Proteus* sp. and *Enterococcus* sp. respectively[Fig 3].

Table 1 Morphological and Biochemical Characterization Test for the identification of bacterial isolates during the study conduct

Isolate No.	Shape	Microscopic		Biochemical											Identification
											TSIA				
		Wet Mount	Gram Staining	Indole	Catalase	MR	VP	Urease	Oxidase	Citrate	Slant	Butt	Gas	H_2S	
A	Rod	P/N	N	N	P	N	N	D	P	P	P/AL	Y/A	N	N	*Pseudomonas*
B	Rod	P	N	N	P	N	N	P	N	P	P/AL	Y/A	P	P	*Proteus*
C	Rod	P	N	P	P				p						*Aeromonas*
D	Rod	P/N	N	N	P	N	N	D	P	P	P/AL	Y/A	N	N	*Pseudomonas*
E	Rod	P	N	N	P	N	N	P	N	P	P/AL	Y/A	P	P	*Proteus*
F	Rod	P	N	P	P				p						*Aeromonas*
G	Cocci	N	P		N				N						*Enterococcus*
H	Cocci	N	P	N	N	N	P			P					*Streptococcus*
I	Rod	P	N	N	P	N	N	P	N	P	P/AL	Y/A	P	P	*Proteus*
J	Rod	P/N	N	N	P	N	N	D	P	P	P/AL	Y/A	N	N	*Pseudomonas*
K	Cocci	N	P	N	N	N	P			P					*Streptococcus*
L	Cocci	N	P		N				N						*Enterococcus*
M	Cocci	N	P	N	N	N	P			P					*Streptococcus*
N	Cocci	N	P	N	N	N	P			P					*Streptococcus*
O	Rod	P/N	N	N	P	N	N	D	P	P	P/AL	Y/A	N	N	*Pseudomonas*
P	Cocci	N	P		N				N						*Enterococcus*
Q	Rod	P	N	N	P	N	N	P	N	P	P/AL	Y/A	P	P	*Proteus*
R	Cocci	N	P	N	N	N	P			P					*Streptococcus*
S	Cocci	N	P		N				N						*Enterococcus*
T	Rod	P/N	N	N	P	N	N	D	P	P	P/AL	Y/A	N	N	*Pseudomonas*

Table 2 Microbial isolates obtained at various concentrations of xenobiotic

Xenobiotic	Concentration	Gram Positive	Gram Negative
Hexane	100 ppm	*Enterococcus* sp	*Aeromonas* sp
	500 ppm	*Enterococcus* sp	*Proteus* sp
Toluene	100 ppm	*Streptococcus* sp	*Aeromonas sp, Proteus sp*
	500 ppm	*Streptococcus* sp	–
Xylene	100 ppm	*Streptococcus* sp	*Aeromonas* sp
	500 ppm	*Enterococcus* sp	*Pseudomonas* sp
Phenol	100 ppm	*Streptococcus* sp	*Pseudomonas* sp
	500 ppm	–	–

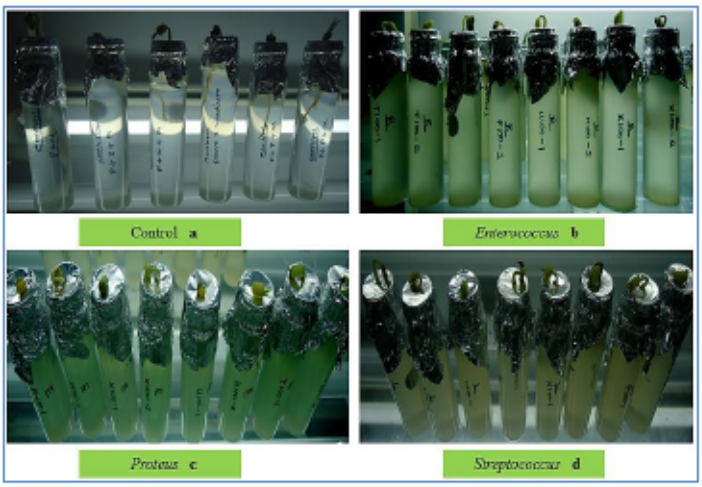


Figure 1 Hydroponically raising seedlings in the presence of xenobiotic and isolated bacteria (test group) a- control (plant & media), b- *Enterococcus*, c- *Proteus*, d- *Streptococcus*

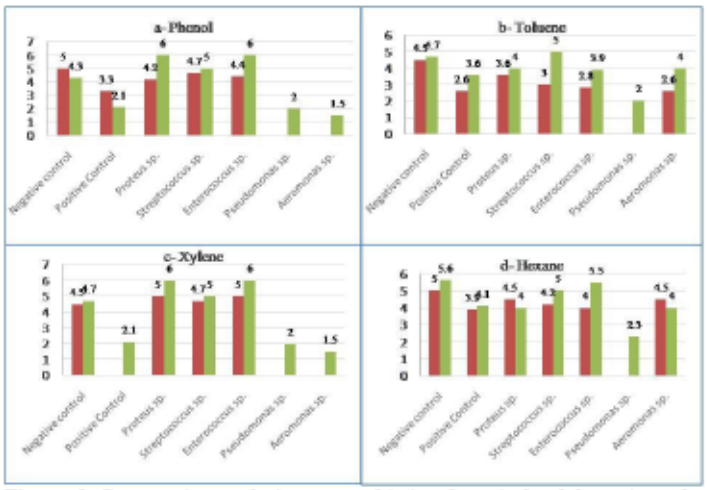
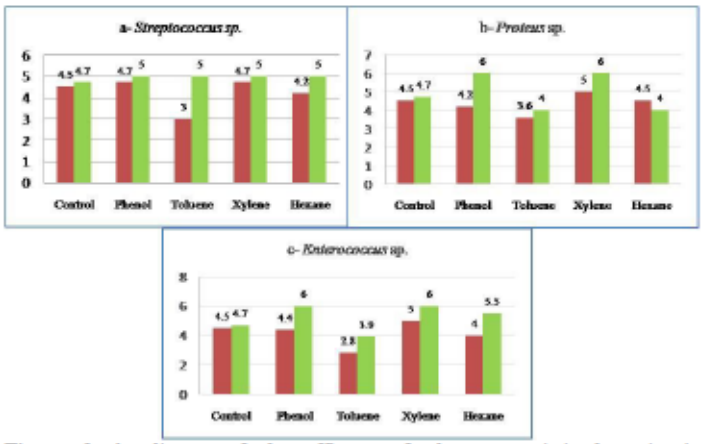


Figure 2 Comparative analysis on xenobiotics from isolated bacteria under hydroponically raised seedling's root shoot length due to: (a) phenol, (b) toluene, (c) xylene and(d) hexane (Red bars- root length (in cms) and Green bars- shoot length (in cms) Negative control: Plant+Medium, Positive control: Plant+Medium+Xenobiotic



Fiureg 3 A glimpse of the efficacy of plant synergistic bacteria (a- *Streptococcus*sp., b- *Proteus*sp., c- *Enterococcus*sp.) on the root-shoot length of the hydroponically grown seedlings(Red bars- root length (in cms) and Green bars- shoot length (in cms)

CONCLUSION

There is a rising demand for the increase in agricultural output to feed the exponentially growing population in India. This comes by compromising with the good agriculture practices such as using organic manure and plant derived pesticides. Due to the increased use of chemical pesticide and fertilisers for an instant benefit, we are disrupting the homoeostasis of the soil biome. The effluents from the industry also directly contribute to this process which in turn elevate the level of xenobiotics and heavy elements in the soil. The worst happens when the land becomes barren due to the persistent use of the chemicals leading to its accumulation and bio magnification in the food chain. The remedial approach for bioremediation, which offers a great promise in future to deal with this problem. Subsequent exploration and designing of microbes capable of degrading spectrum of compounds can be a wise approach to regain the loss of agricultural resources we are incurring now.

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BIOSYNTHESIS OF Mg AND Mn INTRACELLULAR NANOPARTICLES VIA EXTREMO-METALLOTOLERANT *Pseudomonas stutzeri*, $B_{4\ Mg/W}$ and *Fusarium nygamai*, $F_{4\ Mn/S}$

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ABSTRACT

Thirteen microbial isolates were evaluated for green synthesis of Mg and Mn nanoparticles. The isolates were come from soil and wastewater samples from detergent processing industry. Metallotolerance ability of isolates was assessed towards these metals. Bacterial isolate $B_{4\ Mg/W}$ was selected as highly **extremo-tolerant for Mg^{+2}** and can grow between 800 to 15000ppm (80-1500%)and was identified as ***Pseudomonas stutzeri*, $B_{4\ Mg/W}$**. Fungal isolate $F_{4\ Mn/S}$ was selected as **extremo-tolerant for Mn^{+2}** and grow in the range 800 to 45000ppm (80-4500%) and was identified as ***Fusarium nygamai*, $F_{4\ Mn/S}$**. Biosynthesis of the Mg and Mn nanoparticles was achieved in both cases extracellular and intracellular. The nanoparticles were characterized using atomic absorption spectrophotometer (AAS), dynamic light scattering (DLS) and transmission electron microscope (TEM). *Pseudomonas stutzeri*, $B_{4\ Mg/W}$ nanoparticle size was ranges from (229.3-553.2 nm) with different mean number for each size, the maximum mean number 33.7% was that of the particles with size 356.2 r. nm and atomic absorption spectrophotometer (AAS) revealed uptake percentage of the metal was 35.17%. *Fusarium nygamai*, $F_{4\ Mn/S}$ nanoparticles ranges from (61.21-127.5 to 412.5 nm) with different mean numbers for each size, the maximum mean number 23.1% was that of the particle size 82.09 nm and range from 23.1 to 28.1 % and AAS was 27.07%. Antimicrobial activity against *Streptococcus pyogenes* RCMB010015, 31.25 and 62.5 mm followed by *Candida albicans* RCMB05035, 15.63 and 62.5; then *Staphylococcus aureus* RCMB010027 and *Escherichia coli* RCMB010056 gave7.81and 62.5mm for both; while for *Aspergillus fumigates* RCMB02564 gave the least amount of inhibition 1.95 and 15.63mm; moreover *Pseudomonas aeruginosa* RCMB010043 was very resistant for both ***Pseudomonas stutzeri*, $B_{4\ Mg/W}$** and ***Fusarium nygamai*, $F_{4\ Mn/S}$** inracellular nanoparticles, respectively.

Keywords: Antimicrobial activity, extremo- metallotolerance, *Fusarium nygamai*, nanoparticles, *Pseudomonas stutzeri*

INTRODUCTION

Nanotechnology considered nowadays as essential basic science due its importance and involvements in all field of technology **(Rai *et al.*, 2008)**. Every day there is more and more new achievements in the production and characterization of nanomaterials and its applications **(Sharma *et al.*, 2009)**. Synthesis of nanoparticles can be done using several physical and chemical methods but these methods have limitations due to the use of toxic chemicals and controlled conditions as high temperature and pressure **(Rai *et al.*, 2008; Birla *et al.*, 2009; Sau and Rogach, 2010)**. Biological synthesis of nanoparticles has been proved using microorganisms including bacteria **(Husseiny *et al.*, 2007; Shahverdi *et al.*, 2007 & 2009)**, fungi **(Kumar *et al.*, 2007a&b; Parikh *et al.*, 2008; Gajbhiye *et al.*, 2009; Govender *et al.*, 2009)**, actinomycetes **(Ahmad *et al.*, 2003a&b)**, lichens **(Shahi and Patra, 2003)**, algae **(Singaravelu *et al.*, 2007; Chakraborty *et al.*, 2009)**,…etc. It was found that, microorganisms have their own mechanism for production of nanomaterials as secretion of enzymes responsible for reduction of metal ions **(Thakkar *et al.*, 2010)**. The field of nanotechnology improved rapidly and this require more reasonable thinking to make use of the extraordinary characteristics of the produced nanomaterials in the most required applications **(Heiligtag and Niederberger, 2013)**.

The objective of our study was to synthesize magnesium and manganese nanoparticles by metallotolerant microorganisms as benign technique in chance to produce metal nanoparticles with unique properties. Also, characterization of the produced metal nanoparticles was essential. In addition, the produced metal nanoparticles were used as antimicrobial agents as required important application.

MATERIALS AND METHODS

Isolation

The Supplemented Metal-Nutrient (SMN) agar medium which has the same composition as nutrient agar medium with the addition of different concentrations of the metals (Mg^{+2} and Mn^{+2}) separately *viz.* (50, 100, 200 and 500 ppm) was used for the isolation. The medium was poured under aseptic conditions in sterile plates. The plates were inoculated with either 0.1 ml of soil suspension or 0.1 ml of wastewater came from detergents processing wastes from (Savo Factory) in Alamereia, Cairo. The plates were incubated at 30°C for 2 days in case of bacterial isolates and 7 days for fungal isolates.

Extremo- Metallotolerance ability examination of the isolates

The isolates obtained were purified and then, tested for their ability to grow on SMN agar medium containing higher gradient concentrations of the same metal from which it was previously isolated.The maximum concentration was achieved after which no growth was determined.

Biosynthesis of the metal nanoparticles

Supplemented-Metal-Nutrient (SMN) broth medium was prepared and the selected metallotolerant isolates were allowed to grow on sub-lethal concentration of the metal. Incubation was carried out as usual.

Separation of intracellular and extracellular nanoparticles

At the end of the incubation period for bacterial and fungal isolates the extracellular filtrate was separated by centrifugation and filtration and cells were washed with distilled water. The intracellular contents were obtained by ultrasonic disruption of cells with an ultrasonic processor (Cole Parmer Ultrasonic Homogenizer CPX 400) over three 15 s periods, and with an interval of 45 s between periods. The sonicated samples were centrifuged at 15,000 rpm for 30 min at 4°C to remove cell-debris. The supernatants were then used for characterization of intracellular nanoparticles (**Kalimuthu *et al.*, 2008**).

Characterization of metal nanoparticles

The intracellular and extracellular nanoparticles were characterized and examined using atomic absorption spectrophotometer (AAS), dynamic light scattering (DLS). According to the comparison of all the results of metallotolerance ability and DLS measurements, the TEM examination was carried out for the samples giving the most relevant results.

Characterization of the selected bacterial and fungal isolates

The morphological and physiological characteristics of the isolates were studied. The selected fungal isolate was identified genetically based on 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene sequences and the bacterial isolate was identified genetically also based on 16S ribosomal RNA.With A number of the biochemical tests were made using The GEN III MicroPlate test panel.

Studying the antimicrobial activity and MIC determination for the extracellular and intracellular nanoparticles

The antimicrobial activity was done by measuring the minimum inhibitory concentration MIC for the selected intracellular nanoparticles of ***Fusarium nygamai,*** $F_{4\ Mn/S}$ and ***Pseudomonas stutzeri,*** $B_{4\ Mg/W}$ using broth micro dilution method (**Saini *et al.*, 2005**) against *Aspergillus fumigatus (RCMB 02564), Candida albicans* (RCMB 05035), *Staphylococcus aureus* (RCMB 010027), *Streptococcus pyogenes* (RCMB 010015), *Pseudomonas aeruginosa* (RCMB 010043) and *Eschericia coli* (RCMB 010056).

RESULTS AND DISCUSSION

Isolation on SMN agar medium lead to selection of six bacterial magnesium isolates, six bacterial and one fungal manganese isolate (Table 1). A metallo-tolerance ability examination for the selected isolates was performed by increasing metal concentration up to lethal concentration. The most resistant microbial isolates were selected and grow at sub- lethal concentration (Table 2 and 3).

Table 1 Isolation of different microorganisms on media containing Mg^{+2} and Mn^{+2}

Isolate code	Source of isolation	Concentration from which isolate selected (ppm)	Metal used
$B_{1\ Mg/W}$	water	500	Mg^{+2}
$B_{2\ Mg/W}$	water	500	Mg^{+2}
$B_{3\ Mg/W}$	water	500	Mg^{+2}
$B_{4\ Mg/W}$	water	500	Mg^{+2}
$B_{5\ Mg/S}$	Soil	500	Mg^{+2}
$B_{6\ Mg/S}$	Soil	500	Mg^{+2}
$B_{1\ Mn/S}$	soil	200	Mn^{+2}
$B_{2\ Mn/W}$	water	500	Mn^{+2}
$B_{3\ Mn/W}$	water	200	Mn^{+2}
$F_{4\ Mn/S}$	Soil	200	Mn^{+2}
$B_{5\ Mn/W}$	water	200	Mn^{+2}
$B_{6\ Mn/S}$	Soil	500	Mn^{+2}
$B_{7\ Mn/S}$	Soil	200	Mn^{+2}

Where: W means water, S means soil, B means bacteria, F means fungi.

Kaul *et al.* (2012) revealed that, *Pochonia chlamydosporium*, and *Aspergillus fumigatus* were tested to be grow on three different magnesium salts i.e. magnesium sulphate, magnesium chloride and magnesium oxide at two concentrations *viz.* 1000 and 10000 ppm of magnesium salts. The growth of all fungi and bacteria was very poor in the media containing magnesium compounds at 10000 ppm concentration. Comparing to the present study, the metallotolerance examination of the isolates at different gradient elevated concentrations of Mg^{+2} step by step has shown that, there was great ability of the selected four Mg^{+2} bacterial isolates *Viz.* ($B_{2\ Mg/W}$, $B_{4\ Mg/W}$, $B_{5\ Mg/S}$ and $B_{6\ Mg/S}$) to tolerate Mg^{+2} at concentrations up to 15000 ppm (617.157 mM) which equals to 152.11 g/l salt of $MgSO_4.7H_2O$ (152110 ppm). As a result the growth of $B_{4\ Mg/W}$ isolate on magnesium represent as extremo metallotolerant.

On the other hand, one Mn^{+2} fungal isolate ($F4_{Mn/S}$) was selected to grow in a range 800 to 45000ppm (12.3%) as extrme-metalltolerant. Concerning the metalltolerance of Mn^{2+} a metal resistant *Bacillus* sp. strain, isolated from soil was used in an earlier study. The effect of manganese concentration on its growth was monitored at 100 mgl^{-1}, 150 mgl^{-1} or 200 mgl^{-1} manganese (**Sinha *et al.*, 2011**). In addition, **Cheung *et al.* (1982)** reported that, the growth rate of *Bacillus stearothermophilus* cells in a chemically defined medium was inversely proportional to the concentration of Mn^{2+} between 15 and 300 µM. As the Mn^{2+} concentration was increased from 0 to 10 µM the growth was increased proportionally. It was found that, manganese inhibited growth at all concentrations above 15 µM and the optimal Mn^{2+} concentration for growth of vegetative cells was in the narrow range between 10 and 15 µM.

Selection was made according to metallotolerance ability of the microbial isolates, DLS measurements and the easier isolates to manipulate were preferred in selection. The two isolates ($B_{4\ Mg/W}$ and $F_{4\ Mn/S}$) were allowed to grow at 10000 and 30000 ppm of Mg^{+2} and Mn^{+2}, respectively. Both isolates ($B_{4\ Mg/W}$ & $F_{4\ Mn/S}$) were chosen for intracellular nanoparticles production.

Table 2 A summary of the metallo-tolerance ability of the selected Mg isolats

Isolate code	Growth of selected Mg^{+2} isolates												
	Mg^{+2} concentration (ppm)												
	800	1000	1200	1400	1800	2000	3000	5000	7000	9000	11000	13000	15000
$B_{1Mg/W}$	++	+	++++	-ve	++++	++++	-ve	-ve	-ve	-ve	-ve	-ve	-ve
$B_{2\ Mg/W}$	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	++
$B_{3\ Mg/W}$	++++	++++	++++	++++	+++	++	-ve	-ve	-ve	-ve	-ve	-ve	-ve
$B_{4\ Mg/W}$	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	++
$B_{5\ Mg/S}$	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	++
$B_{6\ Mg/S}$	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	++

Table 3 A summary of the metallo-tolerance ability of the selected Mn isolats

Isolate code	Growth of selected Mn^{+2} isolates																	
	Metal concentration (ppm)																	
	800	1000	2000	3000	4000	5000	6000	7000	8000	9000	10000	15000	20000	25000	30000	35000	40000	45000
$B_{1\ Mn/S}$	++++	++++	++++	++++	++++	++++	+++	++	++	+	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
$B_{2\ Mn/W}$	++++	+++	+++	++	++	++	+	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
$B_{3\ Mn/W}$	++	++	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------
$F_{4\ Mn/S}$	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	++	+
$B_{5\ Mn/W}$	+++	++	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------
$B_{6\ Mn/S}$	++++	++++	++++	++++	++++	++++	+++	++	+	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Studying the intracellular nanoparticles samples with AAS has shown that, the isolate $B_{4\ Mg/W}$ is capable of uptake of 35.17% of the used concentration of the metal (12852ppm). The isolate $F_{4\ Mn/S}$ is capable of uptake of only 27.07% of the used concentration of the metal (24860 ppm).

Studying the intracellular nanoparticles samples with DLS has shown that, the size distribution of the nanoparticles in the intracellular sample of isolate $B_{4\ Mg/W}$ ranges from (229.3-553.2 nm) with different mean number for each size, the maximum mean number 33.7% was that of the particles with size 356.2 nm. The size distribution of the intracellular sample of isolate $F_{4\ Mn/S}$ ranges from (61.21-995.1 r. nm) with different mean number for each size, the maximum mean number 23.1% was that of the particles with size 82.09 r. nm, table (4 & 5) , figure (1 &2).

In an earlier study, *Bacillus sp*. (MTCC10650) was reported for the ability to produce manganese oxide nanoparticles intracellularly. The particle size distribution examination showed that the average size of the particles under investigation were 4.6 ±0.14 nm, with some particles, having 6–8 nm size and a very small percentage having diameter greater than 10 nm. The particles were isotropic in nature and monodispersed without any agglomeration. This was further confirmed by the TEM micrograph of the cell taken at higher magnification (**Sinha *et al*., 2011**).

The nanoparticles samples were studied with TEM and it was found that, for the intracellular sample of isolate $B_{4\ Mg/W}$ the metal nanoparticles are spherical in shape. The size of the particles with respect to the spherical form ranges from from 2.90 nm to 9.44 nm. In addition, *Aspergillus flavus* strain TFR-12 was found to have potential to synthesize monodispersed MgO nanoparticles with an average diameter of 5.8 nm, MgO nanoparticles were characterized using dynamic light scattering (DLS). The crystal nature was confirmed by high resolution transmission electron microscope (HR-TEM) (**Raliya *et al*., 2014**).

Hosseinkhani and Emtiazi (2011) reported in an earlier study the ability of *Acinetobacter* sp. isolate to produce extracellular Bixbyite-like Mn_2O_3 NPs. Characterization of morphology, size and chemical structure of these particles was determined by TEM, SEM, XRD and FTIR. The data showed that, this bacterium could produce NPs when grown aerobically in special medium supplemented with 1 mM $MnCl_2$. Studying the morphology and size distribution of the Mn-oxides showed clearly that, the biogenic Mn oxides nanoparticles were less than 500 nm.

Table 4 Dynamic light scattering measurements of intracellular sample of isolate $B_{4\ Mg/W}$ showing size distribution by number (%)

Size nm	Mean Number (%)	St. Deviation Number (%)
229.3	1.3	1.8
265.6	10.0	5.6
307.6	26.3	4.5
356.2	33.7	2.8
412.5	22.3	6.0
477.7	6.3	2.8
553.2	0.2	0.3

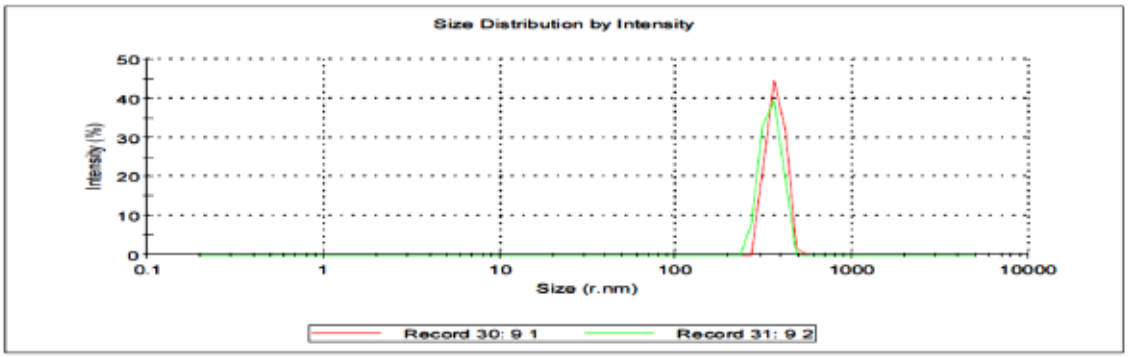


Figure 1 Dynamic light scattering measurements of intracellular sample of isolate $B_{4\ Mg/W}$ showing size distribution by intensity (%)

Table 5 Dynamic light scattering measurements of intracellular sample of isolate $F_{4\ Mn/S}$ showing size distribution by number (%)

Size nm	Mean Number (%)	St. Deviation Number (%)
61.21	3.6	3.2
70.89	13.9	7.2
82.09	23.1	3.9
95.07	21.8	2.2
110.1	12.5	3.6
127.5	4.1	1.9
147.7	0.6	0.4
553.2	4.8	1.7
640.7	5.0	1.9
741.9	3.5	1.2
859.2	1.3	0.4
995.1	0.1	0.0

Raliya and Tarafdar (2014) reported that, magnesium nanoparticles were synthesized using isolated soil fungi by employing various precursor salts of sulfate salts, nitrate salts, chloride salts and oxide salts *viz*. (MgO, $MgSO_4$, $MgCl_2$, $MgNO_3$). It was concluded that, 0.01 mM precursor salt concentration, 72 h of incubation at pH 5.5 and temperature 28 °C resulted smaller nanoparticles obtained. Bio-transformed products were analyzed using valid characterization technique i.e. dynamic light scattering, transmission electron microscopy, atomic force microscopy, energy dispersive X-ray spectroscopy. The average size of Mg nanoparticles was 6.4 nm. Result shows variability in morphological features of biosynthesized nanoparticles.In addition, earlier studies in the green synthesis of magnesium nanoparticles showed that, *Nepheliumlappaceum* L. peels was effectively used for the synthesis of magnesium oxide nanoparticles as a natural ligation agent. The XRD and SEM revealed the crystallinity and spherical morphology of the biosynthesized nanoparticles. The size of the particles was found to be 60-70 nm (**Suresh *et al*., 2014**).

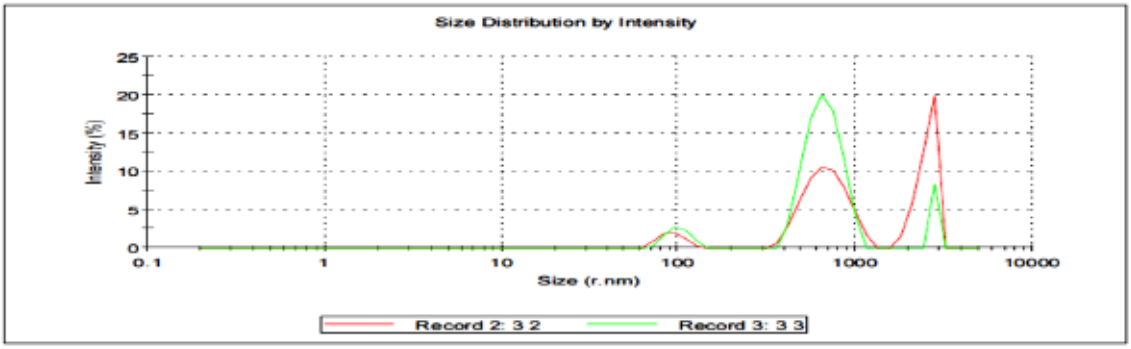
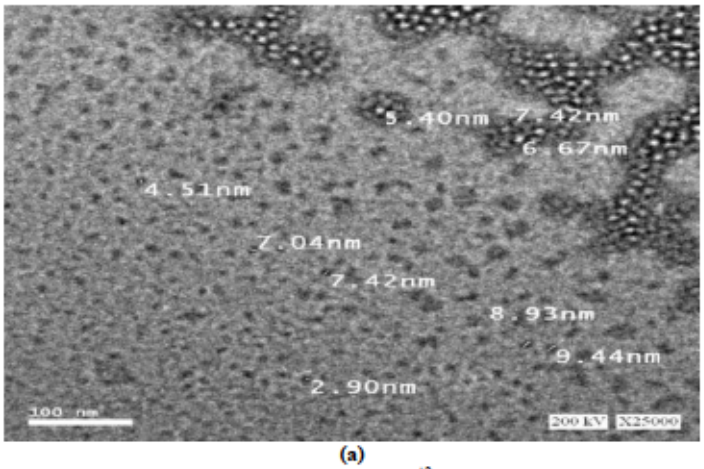


Figure 2 Dynamic light scattering measurements of intracellular sample of isolate $F_{4\ Mn/S}$ showing size distribution by intensity (%)

Waghmare *et al*. (2011) reported the synthesis of manganese nanoparticles by actinomycetes (*Streptomyces* sp.HBUM171191) when exposed to 50 ml of sterilized aqueous solution of manganese sulphate. The formation of whitish yellow to yellow colour indicated the formation of manganese nanoparticles. Transmission electron microscopy results clearly showed the polymorphic nanoparticles with 10 to 20 nm. **Indira and Tarafdar (2015)** reported the synthesis of magnesium nanoparticles by *Aspergillusbrasiliensis* TFR 23 protein. The produced nanoparticles were characterized using appropriate techniques and were having size (< 5.9 nm).

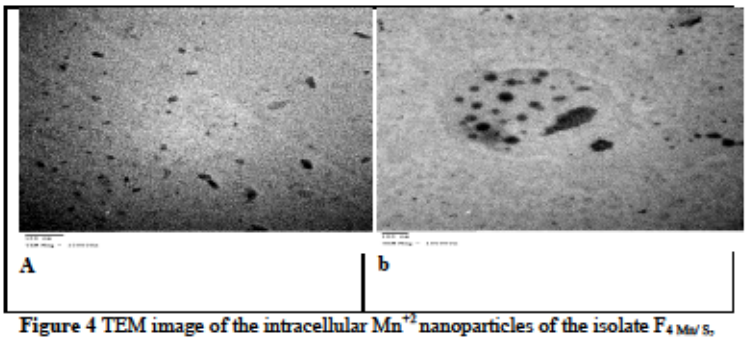
The synthesis of manganese dioxide nanoparticles (MnO_2 NPs) by microorganisms from reducing potassium permanganate was investigated in recent study. The microbial supernatants of the bacterium *Saccharophagus degradans* ATCC 43961 and of the yeast *Saccharomyces cerevisiae* showed positive reactions to the synthesis of MnO_2 NPs. Transmission electron microscopy micrographs revealed the presence of uniformly dispersed hexagonal and spherical particles with an average size of 34.4 nm (**Salunke *et al.*, 2015**).



(a)

Figure 3 a TEM image of the intracellular Mg^{+2} nanoparticles of *Pseudomonas stutzeri*, $B_{4Mg/W}$- Bar scale 100 nm .

The Magnesium nanoparticles were spherical in shape. The size of the NPs ranges from 11.90 nm to 43.29 nm.

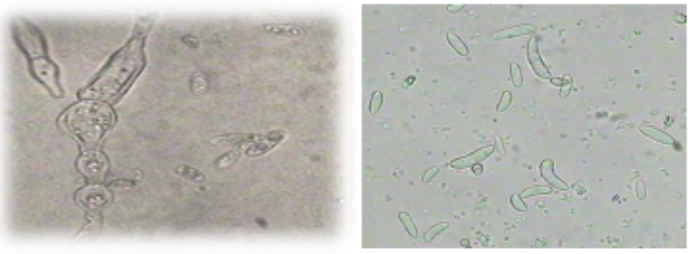


A b

Figure 4 TEM image of the intracellular Mn^{+2} nanoparticles of the isolate $F_{4\ Mn/S}$, (a, b) - Bar scale 100 nm

The manganese nanoparticles showed diversity in shape mostly were spherical. The size of the NPs ranges from 11.90 nm to 43.29 nm.

The $F_{4\ Mn/S}$ isolate identification was based on using Compendium of soil fungi (**Domsch, *et al.*, 1980**), Atlas of clinical fungi (**de Hoog *et al.*, 2000**) &using an Image Analysis System. It was identified also base on genetic characteristics and it was found to be more closely related to *Fusarium nygamai* according to the collected data (Fig. 5), Table (8).



(a) (b)

Figure 5 photographs of Mn^{+2} ($F_{4\ Mn/S}$) on microscopic examination

On the basis of collected data of morphological, physiological and biochemical studies and the comparative study of the properties of $B_{4Mg/W}$ isolate **Bergey's manual of systematic bacteriology, (Krieg, 1984 & Holt *et al.*, 1994) table (6 &7)** and genetic identification also made based on 16S rRNA sequencing, it could be stated that, the present isolate $B_{4Mg/W}$ suggested to be belongs to *Pseudomonas stutzeri*. The present bacterial isolate ($B_{4\ Mg/\ W}$) suggested to be belongs to *Pseudomonas stutzeri* as a new extremotolerant variety. Euclidean distance was 95% between the isolate $B_{4\ Mg/W}$ and *Pseudomonas stutzeri*, While Mn^{+2} fungal isolate $F_{4\ Mn/S}$ extremotolerant variety of *Fusarium nygamai*, *Euclidean distance was* 89% between the isolate ($F_{4\ Mn/S}$) and *Fusarium nygamai* (Fig. 6 & 8).

Table 6 Morphological and biochemical characteristics of the bacterial isolate $B_{4\ Mg/W}$

Characteristic	Result
Morphological characteristics: Shape	rod-shaped have a single polar flagellum
Colonies shape and color	Colonies are disc shaped with ridges radiating from the center
	reddish brown, typically hard, dry and tenaciously coherent
Motility	motile
Aeration	aerobic
Biochemical characteristics	
Gram reaction	gram negative
Catalase	+
Oxidase	+
Utilization of carbon sources	
Dextrin	+
D-Maltose	-
D-Trehalose	-
D-Cellobiose	-
Gentiobiose	-
Sucrose	-
D-Turanose	-
Stachyose	-
D-Raffinose	-
α-D-Lactose	-
D-Melibiose	-
β-Methyl-D-Glucoside	-
D-Salicin	-
N-Acetyl-D-Glucosamine	-
N-Acetyl-D-Galactosamine	-
N-Acetyl Neuraminic Acid	-
α-D-Glucose	+
D-Mannose	-
D-Fructose	+
D-Galactose	-
3-Methyl Glucose	-
D-Fucose	+
L-Fucose	+
L-Rhamnose	-
Inosine	-
D-Sorbitol	-
D-Mannitol	-
D-Arabitol	-
myo-Inositol	-
Glycerol	-
D-Glucose- 6-PO4	-
D-Fructose- 6-PO4	±
Characteristic	**Result**
Pectin	+
D-Galacturonic Acid	-
L-Galactonic Acid Lactone	-
D-Gluconic Acid	+
D-Glucuronic Acid	-
Glucuronamide	+
Mucic Acid	-
Quinic Acid	-
D-Saccharic Acid	-
p-Hydroxy-Phenylacetic acid	-
Methyl Pyruvate	-
D-Lactic Acid Methyl Ester	-
L-Lactic Acid	-
Citric Acid	+
α-Keto-Glutaric Acid	+
D-Malic Acid	-
L-Malic Acid	+
Bromo-Succinic Acid	+
Tween 40	+
γ-Amino-Butyric Acid	-

α-Hydroxy- Butyric Acid	-
β-Hydroxy-D,L- Butyric Acid	-
α-Keto-Butyric Acid	-
Acetoacetic Acid	+
Propionic Acid	-
Acetic Acid	+
Formic Acid	-
Utilization of nitrogen sources	
D-Serine	-
Gelatin	-
Glycyl-L-Proline	-
L-Alanine	+
L-Arginine	-
L-Aspartic Acid	-
L-Glutamic Acid	+
L-Histidine	-
L-Serine	-
L-Pyroglutamic Acid	+
D-Aspartic Acid	-
Growth at different NaCl concentrations (%)	
1	+
4	+
8	-

Table 7 A comparative study of the characteristics of bacterial isolate B4Mg/W, in relation to reference strain *Pseudomonas stutzeri* (Burgey's manual of systematic bacteriology), (Krieg, 1984)

Characteristics	Bacterial isolate B4Mg/W	Reference strain
Morphological characteristics shape	rod-shaped	rod-shaped
Colonies shape and color	Reddish brown, typically hard, dry and tenaciously coherent.	Yellow colonies, typically hard, dry.
motility	Motile	Motile
Aeration	aerobic	aerobic
Biochemical characteristics		
Gram reaction	Gram negative	gram negative
Catalase	+	+
oxidase	+	+
Utilization of		
Glucose	+	+
trehalose	-	-
glycerol	-	+
L- alanine	+	+
lactate	-	+
L-glutamate	+	+
L-malate	+	+
L-arginine	-	-
citrate	+	+
L-histidin	-	-
Citric acid	+	+
D-Sorbitol	-	-
L-Arginine	-	-
L-Histidine	-	-
Growth at 40 & 41 °C	+	+
Hydrolysis of gelatine	-	-
Growth at 4 °C	-	-
Growth at NaCl (12-15%)	-	-
Growth at pH 3.6	-	-

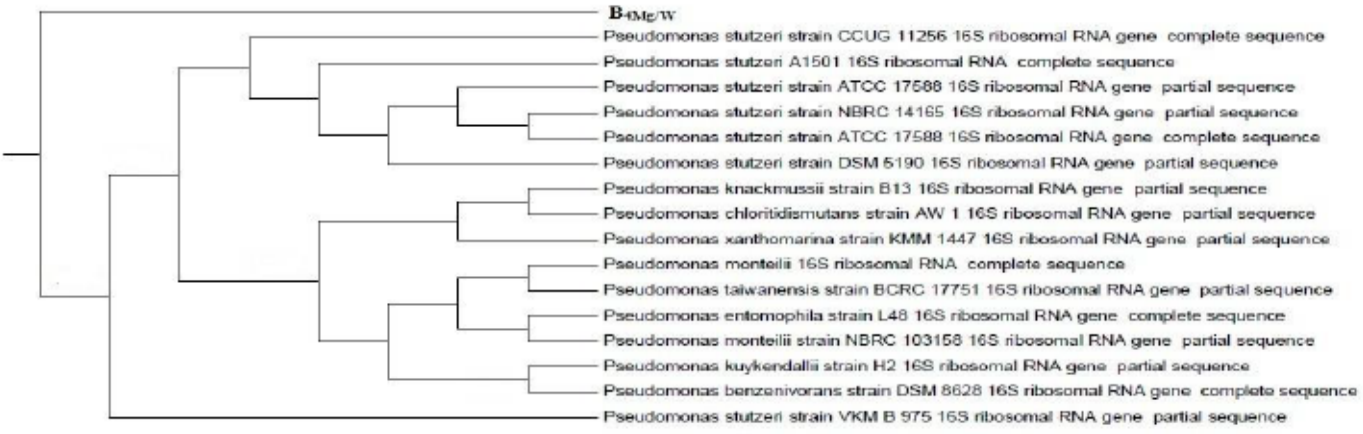


Figure 6 Dendrogram of Mg^{2+} bacterial isolate ($B_{4\ Mg/W}$) and reference *Pseudomonas* strains based on the similarity matrix of phonetic data

Table 8 Morphological and cultural characteristics of the fungal isolate $F_{4\ Mg/S}$

Character	Examination
Culture Exam.:	
Growth characteristics	Colonies on PDA growing rapidly. Mycelium white becoming violet with brown to violet reverse.
Microscopic Exam.:	
Micro-conidia	Micro-conidia, abundant, in short chain or in heads, one celled, fusiform 12X3 µm.
Macro-conidia	Fusiform, 3-5 septate, not abundant, 25X4.0 µm.
Chlamydospores	Chlamydospores, abundant, almost intercalary, single or in chain.

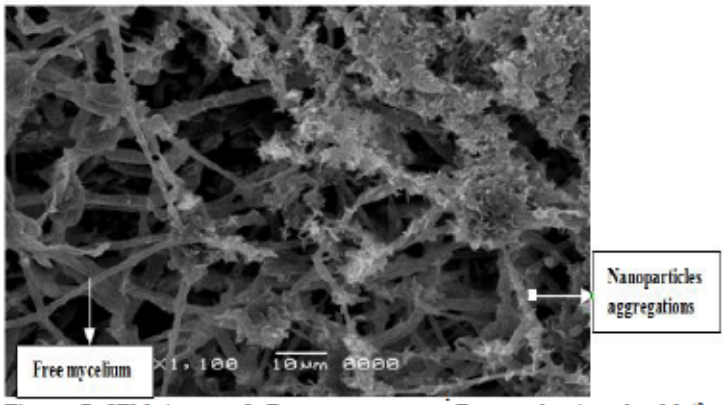


Figure 7 SEM image of *Fusarium nygamai*, $F_{4\ Mn/S}$ showing the Mn^{+2} nanoparticles on the mycelium of the fungus

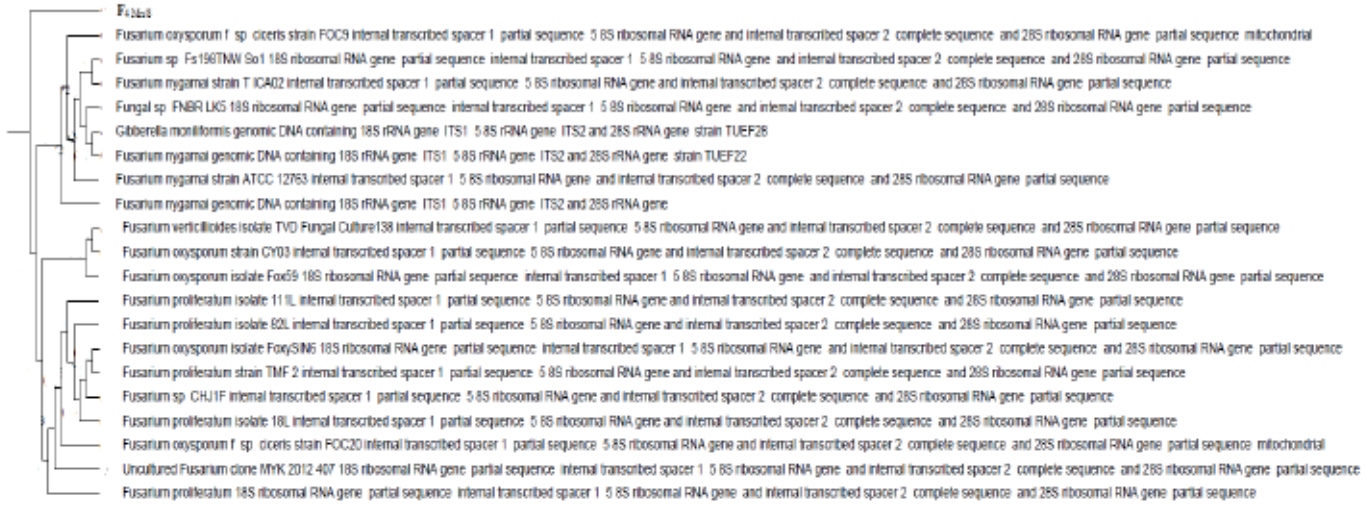


Figure 8 Dendrogram of Mn^{+2} fungal isolate $F_{4\ Mn/S}$ and reference *Fusarium* strains based on the similarity matrix of phonetic data

The antimicrobial activity study of the nanoparticles samples has shown that, all samples have antimicrobial activity against certain test organisms indicated by the inhibition of their growth (table 9).

Table 9 Minimum inhibitory concentration measurements of the produced microbial Mn^{2+} and Mg^{2+} nanoparticles by the most potent metal nanoparticles producers

	Nanoparticle sample		Standard (ppm)
Tested microorganisms	**1**	**2**	
Fungi	**Minimum inhibitory concentration of nanoparticles (ppm)**		amphotericin
Aspergillus fumigatus (RCMB 02564)	15.63	1.95	0.98
Candida albicans (RCMB 05035)	62.5	15.63	3.9
Gram positive bacteria			Ampicillin
Staphylococcus aureus (RCMB 010027)	62.5	7.81	0.015
Streptococcus pyogenes (RCMB 010015)	62.5	31.25	0.06
Gram negative bacteria			Gentamycin
Pseudomonas aeruginosa (RCMB 010043)	NA	NA	62.5
Eschericia coli (RCMB 010056)	62.5	7.81	0.12

Where (1): Intracellular NPs of isolate *Fusariumnygamai*, $F_{4\ Mn/S}$, (2): Intracellular NPs of isolate *Pseudomonas stutzeri*, $B_{4\ Mg/W}$
(NA): means no activity

Antimicrobial activity against *Streptococcus pyogenes* RCMB010015, 31.25 and 62.5 mm followed by *Candida albicans* RCMB05035, 15.63 and 62.5; then *Staphylococcus aureus* RCMB010027 and *Eschericia coli* RCMB010056 gave7.81and 62.5mm for both; while for *Aspergillus fumigates* RCMB02564 gave the least amount of inhibition 1.95 and 15.63mm; moreover *Pseudomonas aeruginosa* RCMB010043 was very resistant for both ***Pseudomonas stutzeri*, $B_{4\ Mg/W}$** and ***Fusarium nygamai*, $F_{4\ Mn/S}$** intracellular nanoparticles, respectively.
Also, **Moustafa *et al.* (2015)** reported that, ***Fusarium nygamai*** was a Zn metallotolerant and nanoparticles producer. The results revealed that, Zn nanoparticles have antimicrobial activity against *Aspergillus fumigatus*, *Candida albicans*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Eschericia coli*.

Tindall *et al.* (1980) stated that, magnesium concentration as low as 10 mM exerts an inhibitory effect on microorganisms isolated from the Dead Sea It has also found that, varied results were obtained among G +ve and G –ve bacteria which may referred to the size of the produced nanoparticles as reported by **Sundrarajan *et al.* (2012).**

CONCLUSION

Manganese (II) nanoparticles have been synthesized by *Fusarium nygamai*, $F_{4\ Mn/S}$ extremo-metallotorent fungus and it was found that, it can produce nanoparticles intracellularly and extraceluellarly. Also, *Pseudomonas stutzeri*, $B_{4\ Mg/W}$ was proved as magnesium (II) nanoparticles producer intracellularly and

extracelluellarly. Only the samples with the best results were chosen. They showed different morphology and size on DLS and TEM examinations. All nanoparticles samples have different high power abilities of antimicrobial activity against test organisms. The green synthesis of the metal nanoparticles was achieved with the possibilities of using these nanoparticles in other serious applications and may have more important impact in other fields.

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MICROBIOLOGICAL QUALITY OF MOROCCAN LABELED *EUPHORBIA RESINIFERA* HONEY

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ABSTRACT

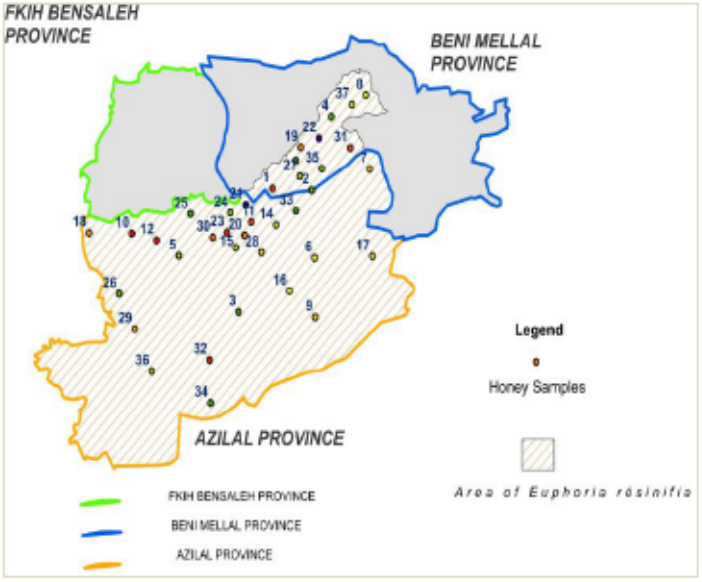
In the present work, microbiological profile of thirty-seven samples of labeled honey were collected in a Protected Geographical Indication "PGI" area of Tadla-Azilal region, which is an endemic zone of *Euphorbia resinifera* plant. A profile was assessed using conventional microbial methods, like enumeration, detection and/or germs identification, in accordance with ISO norms. This is the first study in which a honey with Moroccan "PGI" was tested, in order to assess its compliance with bacteriological recommendations. Coliforms (Total and fecal Coliforms), *Salmonella* spp., *Shigella* spp., *Sporus of Bacillus cereus* and *Clostridium perfringens* were not detected. The numbers of Standard Plate Count "SPC" were less than 10^2 CFU.g^{-1} for all samples. The molds and yeasts were found among samples and 32% and 40% of samples were positive, respectively. However, no samples showed a higher value than recommended limit [10^2 CFU.g^{-1}]. We conclude that samples of labeled euphorbia honey of Tadla-Azilal analyzed present good commercial quality parameters (SPC, molds and yeasts "absence of unwanted fermentations"), a good sanitary quality (absence of coliforms and *S. aureus*) and are safe (*Slam.*, *Shig.*, Sporus of *B. cereus* and *C. perf.*). Standardization (regulation and specifications) and a rationalization of beekeeping techniques throughout *Euphorbia* "PGI" area studied may further sustainably improve the quality of this unique honey, and ensure it over the years.

Keywords: Morocco, labeled *Euphorbia resinifera* honey, Bacteriological Quality

INTRODUCTION

The *Euphorbia resinifera* is one of the specific and endemic plants of Moroccan Atlas Mountains (Picture 1). Generally, the *Euphorbia* plants have high adverse effect level (due to the Latex component, which is a powerful alkaloid), so they has been studied for their antifungal and antibacterial properties (**Kamba *et al.*, 2010; Benmehdi *et al.*, 2013**). In addition, the honeys produced from these plants confirms the antibacterial and antifungal activity (**Malika *et al.*; 2004, Crousilles, 2014; Bouhlali *et al.*, 2016**). Likewise, generally the intrinsic properties of honey (osmolality, pH, hydrogen peroxide, phenolic components and flavonoids) affect the growth and survival of microorganisms by bacteriostatic or bactericidal action. (**Adock, 1912; White *et al.*, 1962; Iurlina and Fritz, 2005; Kačaniová*et al.*, 2009; Adenekan *et al.*, 2010**). Furthermore, the low pH, the low water activity and the high sugar content of undiluted honeys prevent the growth of many species of microorganisms (**Snowdon and Cliver, 1996, Snowdon, 1999**). In consequence, *Euphorbia* honey can be expected to contain a small number and limited varieties of microorganisms. It can be noted that vegetative forms of human disease-causing bacteria have not been found in honey and, as bacteria do not replicate in honey, a high count of vegetative bacteria is indicative of a recent contamination from a secondary source (**Snowden and Cliver, 1996; McKee *et al.*, 2003; Antúnez *et al.*, 2004**).
The microorganisms of interest are those that withstand the concentrated sugar, acidity and antimicrobial character of honey. These microorganisms; indicative of sanitary or commercial quality, include yeasts, molds, coliforms, *Salmonella, Shigella* and some microorganisms such as sporus-forming bacteria, like *Bacillus cereus* (*B. cereus*) and *Clostridium perfringens* (*C. perf.*), which under certain conditions (e.g. germination and growth in a non-heated-treated product) could cause illnesses in humans (**Snowdon and Cliver, 1996; Al-Waili *et al.*, 2012**). Otherwise, the Moroccan standards for honey quality (**Moroccan Norm 08.05.600, 2012**) inspired essentially from Codex Alimentarius Standards (**Codex Stan, 2001**) and the specifications of the label "GPI" (**Moroccan Order, 2012**) includes several chemical and physical parameters but do not require microbiological analysis.
However, the use of adequate hygienic practices during the product handling is required (**Moroccan Law 28-07, 2010; Moroccan Norm 08.0.000, 2008**). In addition, various studies have been carried on the palynological and physicochemical parameters of Moroccan *Euphorbia* honey (**Chakir *et al.*, 2011;Aazza *et al.*, 2014, Terrab *et al.*,2014; Bettar *et al.*, 2015**), but a microbiological contamination has not been extensively investigated.

Figure 1 *Euphorbia resinifera* plant of the "PGI" Tadla-Azilal region of Morocco. *Photo. Moujanni*



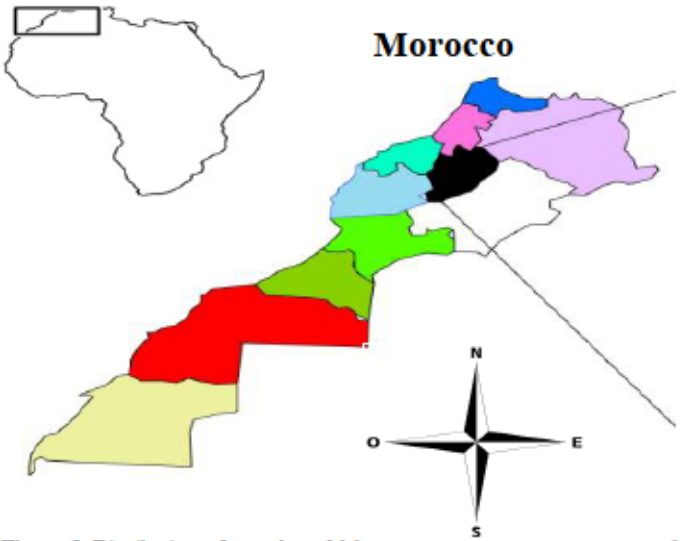


Figure 1 Distribution of samples of labeled monofloral *Euphorbia resinifera* honeys in "PGI" production area of Tadla-Azilal

Euphorbia resinifera honey of Tadla-Azilal region is the first honey labeled "Protected Geographical Indication –PGI-"in Morocco **(Moroccan Order, 2012; ADA, 2014)**. This label was published in the EU Official Journal through the public consultation documents on geographical indications of the Kingdom of Morocco **(European Commission, 2013/C)**.

In this context and in concordance, with the importance and good status of this unique monofloral honey, we decided to investigate about its bacteriological profile targeting the major microbiological contaminants *(SPC, Total coliform, Fecal coliform, Sporus of Bacillus cereus and Clostridium perfringens, Staphylococcus aureus, Salmonella spp, Shigella spp,)* and its fungal profile (*Molds and Yeasts*) which may cause undesirable fermentation. Their counts being indicative of honey's commercial and sanitary quality and safety.

MATERIAL AND METHODS

Sampling

Thirty-seven (37) samples of honey "GPI" mono-floral *Euphorbia resinifera* were supplied directly by the beekeepers of the "PGI" area affiliated to U.C.A.T.AZ cooperative or working individually (Picture 2). Their distribution is indicated in Figure 1.

The samples had not been heated or pasteurized. The productions years of all samples were 2013 and 2014 (Table 1). Upon collection, 250g or 500g of each sample are put in clean commercial labeled container and stored at room temperature pending analysis (Table 1).

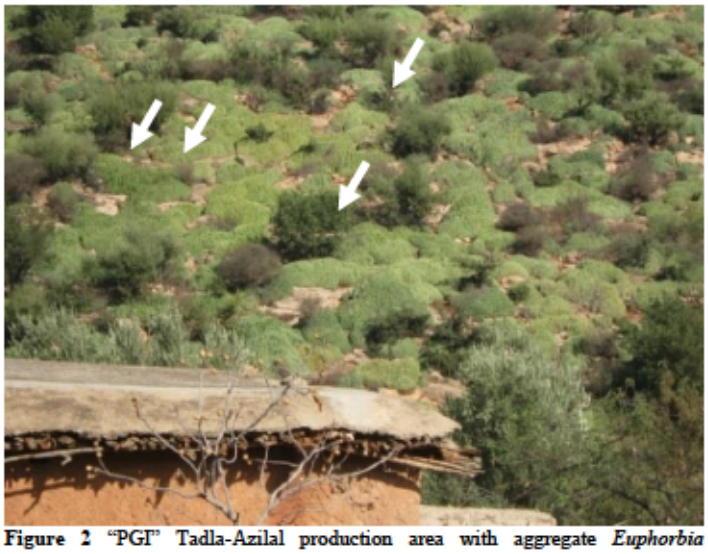


Figure 2 "PGI" Tadla-Azilal production area with aggregate *Euphorbia resinifera* plant

Table1 Information on honey samples studied

Sample	Locality name	Harvest Year
P1	FoumOudi	2014
P2	FoumElaancer	2013
P3	AitMhamed	2013
P4	Tanougha	2013
P5	Tabia	2014
P6	Tabaroucht	2013
P7	Ait Hamza	2013
P8	Elksibah	2013
P9	Tilougguite	2013
P10	Rfala	2013
P11	Afourer	2013
P12	Ben Driss	2012
P13	BeniMellal	2013
P14	Timoulilt	2013
P15	Bin Elouidane-AitOuarda	2013
P16	AitMazigh	2013
P17	Anergui	2013
P18	Bzou	2013
P19	FoumElaancer	2014
P20	AitOuaarda	2014
P21	AitAamir	2014
P22	Tagzirt	2014
P23	Anergui	2013
P24	Afourer	2013
P25	BeniAayat	2014
P26	FoumJemaa	2014
P27	BeniMellal	2014
P28	Azilal	2014
P29	Tanant	2014
P30	Ouaouizaght - Damnat	2014
P31	Tagzirt	2014
P32	AitAbbass -AitMassad	2014
P33	Assaksi - Tagleft	2014
P34	AitBououlli	2014
P35	FoumElaancer -Tagzirt	2014
P36	Ouaoula-AitMhamed	2014
P37	Elksibah	2014

PGI: Protected Geographical Indication
UCATAZ: Union of Beekeepers Cooperative of "PGI" Tadla-Azilal Region, Morocco.

Microbiological analysis.

Ten grams of each sample were mixed with 90mL of Buffered Peptone Water (Biokar) to prepare the initial dilution. This was used at the mother dilution for further serial dilution.

Standard Plate Count (SPC) **(ISO Norm 4833-1, 2013)**: Appropriate serial dilutions (between 10 and 100 colonies per plate) of the samples in the Buffered Peptone Water were placed on standard plate count agar (PCA) (Biokar, France). The plates were incubated at 30°C for 72h.

Coliform counts (TC) **(ISO Norm 4832, 2006)**: Were enumerated on Violet Red Bile Lactose Agar (VRBLA). Plates were incubated at 37°C for 24h.

Fecal coliforms (FC) **(NF Norm V 08 060, 2009):** Were enumerated on Violet Red Bile Lactose Agar (VRBLA). Plates were incubated at 44°C for 24h.
Staphylococcus aureus (*S. aur.)* **(ISO Norm 6888-1, 2003):** Were enumerated on Baird Parker growth medium (Biolife). Plates were incubated at 37°C for 24h and 48h.
Shigella detection (Shig.) **(Lampel KA, 2001):** 25g of the sample was homogenized in 225mL of selenite broth (Biokar) and the volume was transferred to an Erlenmeyer flask and incubated at 35°C for 20h. After this period, a loopful of this broth was plated onto Petri dishes containing XLD agar (Biolife) and *Salmonella–Shigella* agar (SS, Biokar). After incubation for 48h at 35°C, five characteristic colonies of Shigella were biochemically tested on TSI agar (Biokar) and API-20E Biomerieux). The colonies were also serologically tested.
Clostridium perfringens (*C. perf.*) **(ISO Norm 7937, 2004)**: Petri dishes are seeded with a specific quantity of the initial suspension. Other dishes were seeded in the same conditions, using decimal dilutions obtained from the mother suspension. The tryptone sulfite cycloserine (Biolife) was added and then a layer of the same medium is added from above. The dishes were incubated anaerobically at 37°C for 20h. The characteristic colonies are counted. Finally, the characteristic colonies are confirmed and the number of *C. perfringens* per gram of sample is calculated.
Bacillus cereus (*B. cerus.*) **(ISO Norm 7932, 2004)**: Seeding the surface of a solid selective culture medium poured into Petri dishes (MYP Agar) with a specified quantity of the initial suspension. Other dishes were seeded in the same conditions, using decimal dilutions obtained from the mother suspension. The plates are incubated aerobically at 30°C for 18 to 48h. The characteristic colonies are counted and the characteristic colonies are confirmed by hemolysis test and the number of *B. cereus* per gram of sample is calculated.
Salmonella detection (*Salm.*) **(ISO Norm 6579/A1, 2007):** For the detection of the presence of *Salmonella*, 25g of honey sample was homogenized in 225mL of peptone buffered water (Biokar), transferred to an Erlenmeyer flask and incubated at 35°C for 24h. After the incubation period, 1mL was added to a tube containing 10mL of tetra-thionate broth (Biolife). The Rappaport broth (Biokar) received 0.1mL from pre-enrichment and the tubes were incubated at 37°C and 41.5°C for 24h, respectively. After this period, a loopful of each selective broth was plated into Petri dishes containing xylose lysine desoxycholate agar (XLD-Biolife) and CHRO-Magar (Rambach). After incubation for 24h at 37°C, five typical colonies from each agar plate were biochemically tested on TSI agar (Biokar) and API-20E (Biomerieux). The colonies were also serologically tested with polyvalent somatic and flagellar antisera (Probac).
→ *Mold and yeast counts* **(ISO Norm 21527-1, 2008):** Petri dishes prepared using a defined selective culture medium (Glucose Chloramphenicol Agar-Biolife) are seeded. In the number of colonies expected, a specific amount of the initial suspension or decimal dilutions sample / suspension are used. Additional Petri dishes can be seeded in the same conditions; using dilutions decimal obtained from the initial suspension. Plates are incubated aerobically at 25°C for five days. Then, if necessary, the agar plates are allowed to stand in daylight for one to two days. Colonies/propagules are then counted, and if necessary (to distinguish yeast colonies of bacteria colonies), the identity of suspicious colonies is confirmed by examination under the binocular or microscope. The number of yeasts and molds per gram is calculated from the number of colonies/ propagules/germs obtained on Petri dishes selected to dilution ratios to obtain colonies that can be counted. Molds and yeasts are counted separately.

Statistical analysis

All determinations were made in triplicate and the data was processed using XLSTAT, 2015 software.

RESULTS AND DISCUSSION

Results of the microbiological analyzes are given in Table 2. The standard plate count (SPC) also referred to as the aerobic plate count or the total viable count, is one of the most common tests applied to indicate the microbiological quality of food.
The Moroccan legislation **(Moroccan Order, 2004)** does not set values for SPC in honey but establishes only that you follow good hygiene practices in handling and processing of this product because entire microbial load in honey can indicate the possible presence of pathogens **(Moroccan Norm 08.5.600, 2010).** The SPC were isolated from all samples of honey. Their number varied between 10 and 340CFU.g^{-1} with a mean value equal to 76.76±82.93CFU.g^{-1}. This result was inferior to those obtained, for the same type of Moroccan honey, by **Malika *et al.*, (2005).** Compared to other foreign honeys, our results are below Argentinean and French honeys which had main SPC values 244CFU.g^{-1} and 227 CFU.g^{-1} respectively **(Iurlina and Fritz, 2005; Tysset *et al.*, 1981),** while Portuguese commercial honey had better SPC levels [2.10^1CFU.g^{-1}] **(Gomes *et al.*, 2010).** This variation of SPC values could be related to the type of sample, the age and the honey harvest time. In addition, these vegetative forms can be made by secondary contamination, which would also explain the high counts sometimes found in honey **(Snowdon and Cliver, 1996).**

Coliforms (TC and FC) are indicators of fecal contamination and poor hygienic processing conditions. In this study, TC and FC were not detected (level of quantification is 10CFU.g^{-1}) and suggest a respect of good practices for extraction and processing of honey were followed. Our results corroborate with data found by **Rall *et al.*, (2003), Gomes *et al.* 2010, Iglesias *et al.*, (2012), Rios *et al.*, (2014) and Kunovà *et al.*, (2015).** The absence of these microorganisms in analyzed honey was expected since bacteria growth needs water activity more than 0.91 **(Ribeiro and Seravalli, 2004). Snowdon and Cliver, (1996)** already reported that the population of FC in honey varied from 10 to 10^2CFU.g^{-1}. In contrast, in 70 samples of honey analyzed in Nigeria Coliforms and *E. coli* were isolated at rate of 95.7% **(Kokubo *et al.*, 1984).** Also, **Dûmen *et al.*,(2013)** and **Sherwani *et al.*,(2013).**reported respectively that 16% of 80 honey samples were contaminated by coliforms in Turkey and two Pakistanian honey samples over six presents coliforms (0.2×10^1 and 0.4×10^1 CFU.g^{-1})

S. aureus is the causative agent of the numerous outbreaks of foodborne disease worldwide. Poisonings generally occur after an intake of enterotoxins through the alimentary track. The absence of this bacterium in this study constitutes another sanitary index in favor to the quality of this product. In a similar study done on Turkish honeys, 13.4% of the 67 samples analyzed contained *S. aureus* **(Dûmen *et al.*, 2013).**

B. cereus and *C. perfringens*, as producers of spores, are considered as health indicator including uncontrolled land-based, environmental or human contamination. High levels of *B. cereus* in honey constitute a risk to the consumer, as ingestion of 10^5 spores can result in food-borne illness **(Stenfors *et al.*, 2008).** The results of this study demonstrate a negative result regarding detection of sporus of *B. cereus* and *C. perfringens*. However, **Pucciarelli, (2014)** found the incidence of *Clostridium and Bacillus* (42.85 and 39% respectively) in yateí honey from Argentina. In addition, **Ragazani *et al.*, (2008)** studying honey marketed in several Brazilian states found 11% were *Clostridium* genus and 28% of the genus *Bacillus*. **Erkan *et al.*,(2015) and Sherwani *et al.*,(2013)** reported respectively $5.5\times10^1\pm6.3\times10^1$ *B. cereus* mean count in Turkish honey and presence of *B. cereus* in all (six) samples of Pakistanis (Karachi) honey tested.
In respect to safety, none of the 37 samples contained *Salmonella* and *Shigella*. The absence of these pathogens was expected since in addition to its antibacterial properties, honey has low water activity and a pH, which are not in favor to the development of such bacteria **(Snowdon and Cliver, 1996; Alves et al., 2015; Matuella and Torres, 2000).**
In the same way, study of the microbiologic quality of honey samples produced in the surroundings of a large garbage dump in Brazil showed the absence of *Salmonella*. These results confirm the conclusion of Anses that the *Salmonella* survival duration in honey does not exceed one month **(Anses, 2012).**

Table 2 Distribution of microorganisms detected in "GPI" Moroccan *Euphorbia resinifera* honeys

N°	Microorganisms count (CFU.g^{-1})						Per 25g		Sporus	
	SPC	TC	FC	*S.aur*	Yeasts	Molds	*Salm.*	*Shig.*	*C.perf*	*B.cereus*
P1	50	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
P2	340	<10	<10	<10^2	10	<10	Abs	Abs	<10	<10
P3	320	<10	<10	<10^2	70	90	Abs	Abs	<10	<10
P4	270	<10	<10	<10^2	20	<10	Abs	Abs	<10	<10
P5	250	<10	<10	<10^2	93	60	Abs	Abs	<10	<10
P6	60	<10	<10	<10^2	10	<10	Abs	Abs	<10	<10
P7	90	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
P8	30	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
P9	50	<10	<10	<10^2	30	10	Abs	Abs	<10	<10
P10	70	<10	<10	<10^2	30	10	Abs	Abs	<10	<10

P11	40	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
P12	30	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
P13	40	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
P14	10	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
P15	110	<10	<10	<10^2	20	20	Abs	Abs	<10	<10
P16	30	<10	<10	<10^2	10	<10	Abs	Abs	<10	<10
P17	80	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
P18	20	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
P19	30	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
P20	70	<10	<10	<10^2	50	10	Abs	Abs	<10	<10
P21	60	<10	<10	<10^2	20	10	Abs	Abs	<10	<10
P22	40	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
P23	120	<10	<10	<10^2	80	20	Abs	Abs	<10	<10
P24	10	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
P25	70	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
P26	10	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
P27	50	<10	<10	<10^2	50	<10	Abs	Abs	<10	<10
P28	50	<10	<10	<10^2	20	<10	Abs	Abs	<10	<10
P29	10	<10	<10	<10^2	<10	10	Abs	Abs	<10	<10
P30	20	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
P31	50	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
P32	50	<10	<10	<10^2	<10	10	Abs	Abs	<10	<10
P33	50	<10	<10	<10^2	<10	10	Abs	Abs	<10	<10
P34	10	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
P35	60	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
P36	110	<10	<10	<10^2	60	20	Abs	Abs	<10	<10
P37	80	<10	<10	<10^2	<10	<10	Abs	Abs	<10	<10
Mean	76.76	<10	<10	<10^2	38.20	23.33	n.a.	n.a.	<10	<10
SD	82.93	n.a.	n.a.	n.a.	27.20	25.35	n.a.	n.a.	n.a.	n.a.
Min	10	n.a.	n.a.	n.a.	10	10	n.a.	n.a.	n.a.	n.a.
Max	340	n.a.	n.a.	n.a.	93	90	n.a.	n.a.	n.a.	n.a.

n.a.: not applicable- Abs: Absence
Level of quantification = 10^2 CFU.g^{-1} for S. aureus
Level of quantification = 10 CFU.g^{-1} for all microorganisms tested
Level of quantification = 10 sporus for C.perf. and B.cereus

Table 3 Comparing the results of molds and yeasts counts in honey from different countries

References	Nb. samples	Molds and yeast Count CFU.g^{-1}			Country	Incidence Positives/Total analyzed	Limit recommended CFU.g^{-1} [a] [55]
		Min	Max	Mean			
Iurlina and Fritz (2005)	23	0	4.7×10^2	1.64×10^2	Argentina	59%	1.0×10^2
Finola *et al.*, (2007)	23	<1.0×10^1	<1.0×10^1	-	Argentina	-	1.0×10^2
Rios *et al*, (2014)	58	-	-	-	Argentina	17% (7%)	1.0×10^2
Pucciarelli *et al.*, (2014)	28	1.2*	4.7*	3.02*	Argentina	-	1.0×10^2
Rall *et al.*, (2003)	100	<1.0×10^2	1.5×10^5	-	Brazil	64%	1.0×10^2
Sereia *et al.*, (2010)[b]	11	1.9×10^2	1.1×10^3	5.3×10^2	Brazil	-	1.0×10^2
Sereia *et al.*, (2010)[c]	6	1.8×10^1	2.5×10^2	1.0×10^2	Brazil	-	1.0×10^2
Pontara *et al*, (2012)	12	<1.0×10^1	<1.0×10^1	-	Brazil	-	1.0×10^2
Ananis *et al.*, (2013)	35	<1.0×10^1	5.0×10^2	-	Brazil	45.71%	1.0×10^2
Alves *et al.*, (2015)	15	2.2×10^7	3.4×10^7	-	Brazil	20%	1.0×10^2
Giraldo *et al.*, (2013)	7	0	0	0	Colombia	0	1.0×10^2 [d]
Mahmoudi *et al.*, (2016) [e]	34	-	-	-	Iran	5.8%-32.3%	-
Ayansola, (2012) [f]	108	1.0×10^1	2.0×10^3	-	Nigeria	-	-
Ummulkhair, (2014)	15	1.0×10^4	1.2×10^5	-	Nigeria	26.66%	-
Malika *et al*, (2005)	10	<1.0×10^1	3.0×10^1	-	Morocco	30%	-
Present study (yeasts)	37	1.0×10^1	9.3×10^1	3.82×10^1	Morocco	32%	-
Present study (molds)	37	1.0×10^1	9.0×10^1	2.33×10^1	Morocco	40%	-
Sherwani, (2013)	6	0	<1.0×10^1		Pakistan	20%	-
Różańska and Osek (2012)	245	<5.0×10^1	8.0×10^4		Poland	-	1.0×10^2
Gomes *et al.* (2010)	5	1.1×10^1	2.1×10^1		Portugal	60%	1.0×10^2
Feás *et al.*, (2010)	45	1.0×10^1	8.0×10^1	2.2×10^1	Portugal	100%	1.0×10^2
Duman Aydin *et al.*, (2008)	20	1.0×10^2	1.0×10^3		Turkey	40%	1.0×10^2

Dûmen *et al.*, (2013)	500	-	-	-	Turkey	16%-32%	-
Erkan *et al.*, (2015) [g]	50	1.0×10^{2}	1.2×10^{3}	3.5×10^{2}	Turkey	26%	-
Erkan *et al.*, (2015) [h]	50	7.4×10^{3}	1.4×10^{5}	5.4×10^{4}	Turkey	46%	-

**Count in log CFU.g^{-1}*
a: The value recommended by MERCOSUR (Agreement on the Southern Common Market) and CNEVA.
b: Organic honey
c: Inorganic honey
d: The value specified by Colombian Resolución N°1057
e: Count of fungi (Aspergillus, Penicillium, candida and other yeasts)
f: Count of total heterotrophic fungi
g: Count for Mold vegetative form
h: Count for Yeast vegetative form

The results obtained for standard counting of molds and yeasts showed that 32% and 40% of samples were positive respectively for molds and yeasts.
However, the detection of yeasts and molds remains at low levels ([1.0×10^{1}-9.3×10^{1}CFU.g^{-1}] for yeasts with mean=$3.82\times10^{1}\pm2.72\times10^{1}$CFU.g^{-1} and [$1.0\times10^{1}$-$9.0\times10^{1}$CFU.g^{-1}] for molds with average $2.33\times10^{1}\pm2.53\times10^{1}$CFU.g^{-1}). Withal, note that no result exceeds the recommended threshold for yeast (10^{2} CFU.g^{-1}), nor the fermentation honey line (5.0×10^{2} CFU.g^{-1}) **(Fléché *et al.*, 1997).** Furthermore, total most and yeast counts can vary greatly, typically between 0 and 10^{5} CFU.g^{-1}, although high counts are not palatable because of the increased rate of fermentation and the honey is unlikely to pass quality control (changing the taste and the flavor of honey) **(White, 1975).** From that point a view, a few hundred CFU.g^{-1} of yeast are more likely to be found in honey samples.
Table 3 below gives a comparison of the values found in several studies, conducted in the world relating to molds and yeasts in honey. It appears from reading the table that our results are close to those of **Malika *et al.*, (2005), Gomes *et al.*, (2010) and Feás *et al.*, (2010). Finola *et al.*, (2007) and Giraldo *et al.*, (2013)** reported no or low values (<1.0×10^{1}CFU.g^{-1}) of molds and yeasts for respectively Moroccan, Portuguese, Argentinian and Colombian honeys.
However, **Różańska and Osek, (2012)** from Poland, **Mahmoudi *et al.*, (2016)** from Iran, **Rall *et al.*, (2003)** from Brazil, **Erkan *et al.*, (2015)** from Turkey, **Ummulkhair, (2014)** from Nigeria and other authors reported a higher counts of molds and yeasts (Table 3).

CONCLUSION

At the end of this study, it was observed that *Euphorbia resinifera* honey has an acceptable microbiological profile. In fact, none of the analyzed sample contained any microorganisms that have an impact on human health. Additionally, the low levels of microbial contamination associated to a very low rate of mold and yeast indicate that this honey does not undergo any significant degradation and, therefore, this product always keeps its commercial quality. However, it is has to be emphasized the importance of continuous monitoring throughout the honey processing, to ensure the marketing of a reliable food. It is also recommended for governments and producers, to ensure a continuous control and to set up specification conditions during storage (moderate temperatures, increased humidity, granulation of the honey and elevated yeast counts).
Finally, standardization, by national and/or "PGI" specifications, of microbial contamination limits is very important to further improve the quality of honey, and ensure it sustainability over the years.

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IN VITRO ANTIOXIDANT EFFICACY OF EPS OBTAINED FROM *MICROCOCCUS LUTEUS* SNIST- CM 02: A BRIEF STUDY

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ABSTRACT

The polysaccharides biosynthesized by the microorganisms had emerged as an important class of bioactive compound from the last two decades. In the present study, an attempt was taken to evaluate the *in vitro* antioxidant activity of EPS produced by *Micrococcus luteus* SNIST-CM02. The scavenging assays involving 1, 1-diphenyl-2-picrylhydrazyl (DPPH), superoxide radicals, hydroxyl radicals, chelating ability and inhibition effect of lipid peroxidation, for the biosynthesized EPS were studied in order to evaluate the *in vitro* antioxidant activity. The EC50 value of EPS for DPPH, superoxide radicals and hydroxyl radicals was found to be 8.11 mg/mL, 8.00 mg/mL and 10.25 mg/mL respectively. The EPS had a lower inhibition effect of lipid peroxidation than that of ascorbic acid and butylated hydroxytoluene. At a concentration of 12.0 mg/mL, the inhibition was only 70.00%. These results suggest that the EPS synthesized by *Micrococcus luteus* SNIST- CM02 can be used as a good potential antioxidant and can be added to different types of food as a novel antioxidant.

Keywords: Antioxidant; DPPH; Scavenging assay; *Micrococcus luteus*; EPS; EC_{50} Value

INTRODUCTION

The free radicals cause the harmful effect to living system either by the onset of degenerative diseases or by causing the ageing phenomena very fast (**Cross *et al.*, 1987; Beckman and Ames 1998**). Generally, these phenomena are known as oxidative stress (**Michiels *et al.*, 1994**). There are so many compounds which present in the living cell, significantly cease this oxidative stress and these all are collectively known as antioxidant. Although, the cells have its own mechanism to protect itself from the stress, but in some cases the indigenous or exogenous sources saddle of the free radicals lead to the imbalance the redox potential of the living cell. In order to overcome this, the cells need the other alternative antioxidant from outside as a supplement. Therefore, naturally occurring antioxidant molecules have drawn a great attention in the field of medicine and food. Recently, the polysaccharide produced from the microorganism has been explored as the novel antioxidant (**Michiels *et al.*, 1994; Tiwari 2001**).

Generally, EPS synthesized by microorganisms are non-toxic, non-immunogenic, non-carcinogenic and biocompatible in nature. Different types of application related to the exopolymers are like food packaging, capsule coating, gene delivery, drug delivery, molecular chaperon synthesis have been carried out by various authors (**Duan *et al.*, 2007; Gheorghe *et al.*, 2008; Yoshihiro *et al.*, 2010**).

The EPS is biosynthesised in the cytoplasm and then secreted into the extracellular environment facilitating its recovery (**Li *et al.*, 2006**). The biosynthetic pathways and its real mechanism of synthesis of various EPSs are not clearly understood. Few years back, some authors (**Duan *et al.*, 2007**) have proposed a biosynthesis pathway model for the EPS synthesis. The exopolysaccharide bio-synthesized by microorganisms, including bacteria, moulds and yeast, represent an unexploited market (**Sutherland, 2001**). But at the same time, the limiting factor of EPS production by microorganisms is linked to its production cost. The main costs consist of the price of the carbon and nitrogen sources in certain cases (**Donot *et al.*, 2012**).

In the present study, the antioxidant activity of the crude exopolysaccharide (EPS) from a newly isolated strain of *Micrococcus luteus* SNIST- CM02 (screened from the natural habitat) was studied. Carbohydrate based antioxidants are having better applicability over other antioxidants as they are neutral, water soluble and non-toxic to the living cells.

MATERIALS AND METHODS

Microbial strain, culture conditions and eps production

The production and characterization of EPS was performed in our previous study (**Mishra *et al.*, 2016**) from the isolate of *Micrococcus luteus* SNIST- CM02. In this process, EPS was produced in the 250mL Erlenmeyer flask. The composition of the medium for the production of EPS was as follows: Sucrose 60.0 g, K_2HPO_4 7.5 g, NaCl 1.5 g, $MgSO_4.7H_2O$ 0.4 g, Yeast Extract 0.4 g and distilled water 1,000 mL. The pH of the medium was adjusted to 6.5 by adding 0.1 M NaOH. After preparation and sterilization of the medium, 1 mL of the prepared inoculum was inoculated to 100 mL of the production medium and was incubated for 6 days under agitation (150 rpm) at 25°C. The microorganism was maintained on Nutrient agar at 4°C and sub-cultured every 2 weeks. The isolated strain was inoculated to the production medium (Sucrose 60.0 g, K2HPO4 7.5 g, NaCl 1.5 g, MgSO4.7H2O 0.4 g, Yeast Extract 0.4 g and distilled water 1000 mL) and was incubated for 7 days under agitation (150 rpm) at 28 °C. The recovery process for the EPS production was carried out first by removing the cells by centrifugation at 7,000 rpm, for 10 min followed by its precipitation with adding twice the volume of cold isopropyl alcohol (**Singh *et al.*, 2009**).

Preparation of exopolysaccharide (EPS)

Crude EPS was purified by dissolving the precipitates with the water. Further, the solution of the EPS was reprecipitated with ice-chilled isopropyl alcohol. This methodology was followed up to attainment of the 3rd fraction. Each fraction of EPS obtained after the precipitation was made to wash with the acetone before reprecipitation. The pure EPS obtained in 3rd fraction was further subjected to dialysis in order to analyze the *in-vitro* antioxidant activities.

IN VITRO ANALYSIS OF ANTI-OXIDANT ACTIVITY OF EPS

Scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals

The antioxidant analysis was carried out by using 1 mL of EPS solutions was made at different concentrations (0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0 and 16.0 mg/mL) and was added to 4.0 mL of 0.004% solution of DPPH in ethanol. After 30 min, the absorbance was measured at 517 nm. Scavenging ability (%) of DPPH radicals = $\{1- (A_{sample} / A_{control})\} \times 100$, where $A_{control}$ is the absorbance of control, and A_{sample} is the absorbance in the presence of the tested samples. The ascorbic acid (Vitamin C) and butylated hydroxytoluene were taken as positive controls for this study (**Shimada *et al.*, 1992; Guo *et al.*, 2010**).

Scavenging of superoxide radicals

Antioxidant analysis was further analysed by adding, 4 mL of Tris–HCl buffer (pH 8.2) and 1 mL of EPS sample solution with different ranges of concentrations (0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0 and 16.0 mg/mL) were incubated at 30 °C for15 min, followed by the addition of 200 µL of pyrogallol to the mixture, and the reaction was allowed to proceed for 4 min. The reaction was made to stop by adding 0.5 mL of HCl. The absorbance of the mixture was measured at 320 nm. Scavenging ability (%) of superoxide radicals = {1- (A_{sample} / $A_{control}$)} ×100, where $A_{control}$ is the absorbance of control, and A_{sample} is the absorbance in the presence of the tested samples. The ascorbic acid (Vitamin C) and butylated hydroxytoluene were taken as positive controls for this study **(Marklund and Marklund, 1974).**

Hydroxyl radical scavenging activity

The reaction mixture containing EPS (0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0 and 16.0 mg/mL) was incubated with deoxyribose (3.75 mM), H_2O_2 (1 mM), $FeCl_3$ (100 mM), EDTA (100 mM) and ascorbic acid (100 mM) in potassium phosphate buffer (20 mM, pH 7.4) for 60 min at 37 °C **(Halliwell *et al.*, 1987).** The reaction was terminated by adding 1 mL of thio-barbituric acid (1%, w/v) and 1 mL of trichloroacetic acid (2%, w/v) following the incubation in a boiling water bath for 15 min. The absorbance of the mixture was measured at 535 nm against reagent blank. The Scavenging ability (%) of hydroxyl radicals = {1- (A_{sample} / $A_{control}$)} ×100, where $A_{control}$ is the absorbance of control, and A_{sample} is the absorbance in the presence of the tested samples. The ascorbic acid (Vitamin C) and butylated hydroxytoluene were taken as positive controls for this study.

Chelating ability of eps on ferrous ion

The chelating ability of EPS with the ferrous ion (Fe^{2+}) was studied according to the protocol given by **Decker and Welch (1990)**. The Fe^{2+} chelating ability of EPS from *Micrococcus luteus* SNIST- CM02 was studied by measuring the FerroZine iron complex at 562 nm. Here the EDTA was used as a positive control.

Inhibition effect of lipid peroxidation

The antioxidant analysis was finally confirmed by the standard method as given by Kimuya et al. (1981) for effect of lipid peroxidation was followed in which 0.8 mL of egg yolk mixed with 0.1 mol/L phosphate buffer (pH 7.45) and resulted homogenate was added to 0.5 mL of EPS sample solution with different concentrations (0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0 and 16.0 mg/mL). In order to initiate the lipid peroxidation process, 0.5 mL of 25 mmol/L of $FeCl_2$ was added to the above mixture. After incubation at 37°C for 1 hour, 1.0 mL of 20% (w/v) trichloroacetic acid and 1.0 mL of 0.8% (w/v) thiobarbituric acid were added to stop the reaction. The mixture was heated at 100 °C for 20 min, and centrifuged (2400g) for 10 min. The upper layer was collected, and the absorbance was taken at 532 nm. The inhibition (%) effect= {1- (A_{sample} / $A_{control}$)} ×100 was calculated, in which $A_{control}$ is the absorbance of control and A_{sample} is the absorbance in the presence of the tested samples. The Ascorbic acid (Vitamin C) and Butylated hydroxytoluene were taken as positive controls for this study.

RESULTS

***In vitro* anti-oxidant activity of EPS**

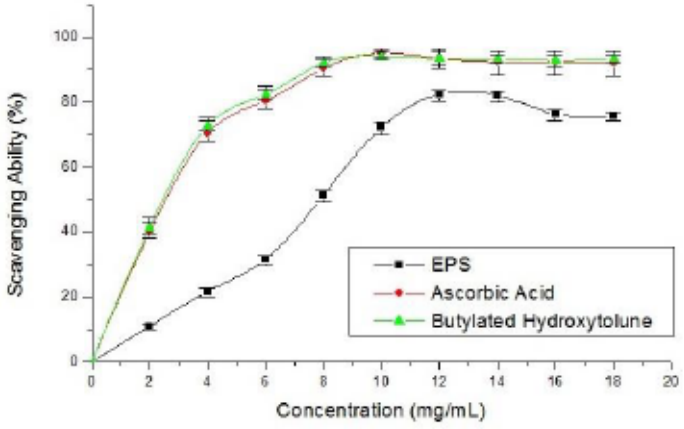

Figure 1 Scavenging of DPPH radicals (Values were representative of three separated experiments)

The *in-vitro* antioxidant property of the EPS had been assessed with DPPH scavenging, superoxide radicals scavenging and lipid peroxidation inhibition assays. These antioxidant activities were compared with those of ascorbic acid and butylated hydroxytoluene. Less scavenging of DPPH and superoxide radicals was observed with EPS than with ascorbic acid and butylated hydroxytoluene at concentration < 12.0 mg/mL, but at the same time the scavenging ability was similar to those of ascorbic acid and butylated hydroxytoluene at concentration ≥ 12.0 mg/mL as found in figure 1 and figure 2.

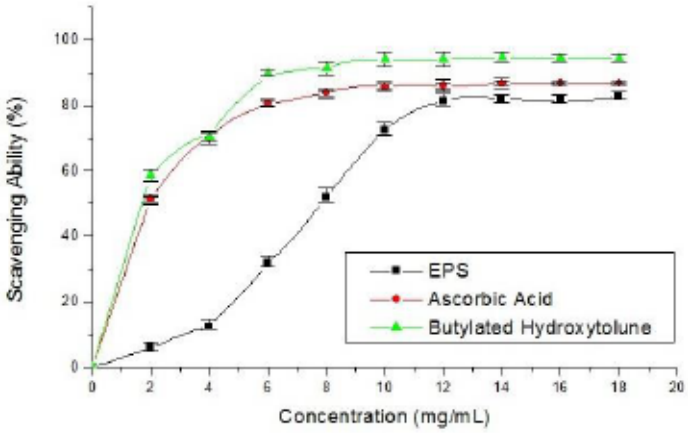

Figure 2 Scavenging of superoxide radicals (Values were representative of three separated experiments)

The scavenging efficacy of the hydroxyl radicals for EPS was found to be same with ascorbic acid and butylated hydroxytoluene at concentration ≥ 8.0 mg/mL. It was observed that, the scavenging ability of EPS sharply increased when the concentration changed from 8.0 mg/mL to 12.0 mg/mL as shown in figure 3.

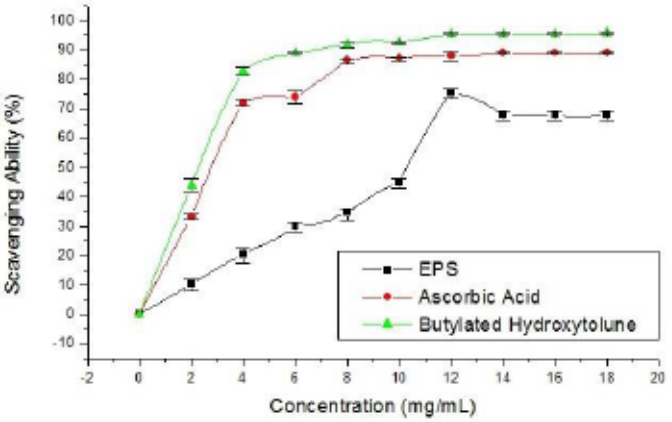

Figure 3 Scavenging of hydroxyl radicals (Values were representative of three separated experiments)

The chelating activity for EPS was also estimated and it was found nearly equal to 70 % at concentration ≥ 6.0 mg/mL of EPS as shown in figure 4. The chelating ability of EPS was lesser than that of EDTA. Previously the chelating activity of some of the carbohydrates like sulfated polysaccharides, acetylated polysaccharides and phosphorylated polysaccharides from *Ramulus mori* was found to be 74.5%, 67.2% and 58.7% at 2.1 mg/mL, respectively **(Zhang *et al.*, 2008).**

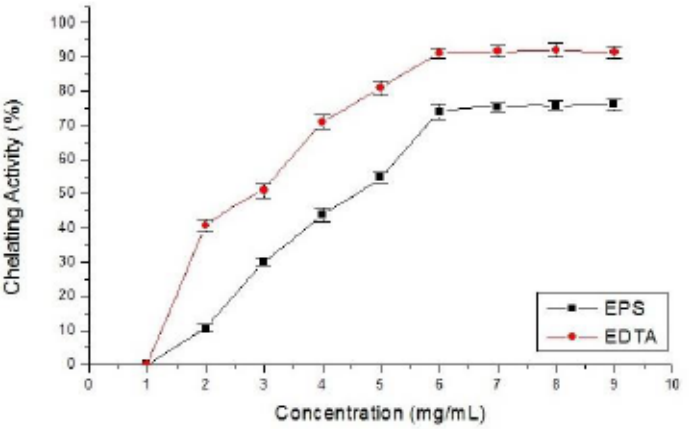

Figure 4 Chelating ability of EPS with ferrous ion (Values were representative of three separated experiments

The EC_{50} value of EPS for DPPH radical is 8.11 mg/mL and for superoxide radical it is 8.0 mg/mL. For the hydroxyl radicals, the EC_{50} value was found to be

10.25 mg/mL. The EPS had a lower inhibition effect of lipid peroxidation than that of ascorbic acid and butylated hydroxytoluene. At the concentration of 12.0 mg/mL, the inhibition was 70.0% as shown in figure 5. These results suggest that the EPS synthesized by *Micrococcus luteus* SNIST- CM02 can be used as a good potential antioxidant.

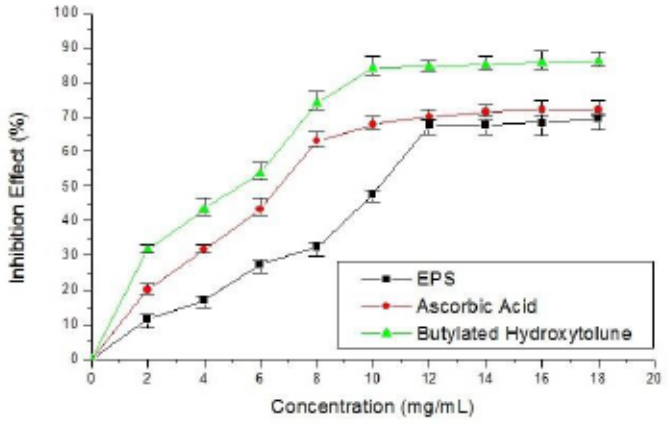

Figure 5 Lipid peroxidation Inhibition (Values were representative of three separated experiments)

DISCUSSION

The results of the present findings depict that EPS from *Micrococcus luteus* SNIST- CM02 have good antioxidant and antiradical activities. From the different types of experiments, it was found that EPS exhibited the concentration dependent antioxidant activity. The antioxidant activity of the microbial EPS probably related to the monosaccharide component, molecular size, conformation and due to its bioactivity. It has been reported that the bioactivities of polysaccharides are closely associated with the type of glycosyl units, the configuration of glycosidic bonds, and the substituents of the polysaccharides. Additionally, the spatial structure and relative molecular mass of polysaccharides conjointly have an effect on the bioactivity **(Tsiapali *et al.*, 2001).** Therefore, the antioxidative activity of the EPS is not the results of any single factor. It is the results of several factors combined within the variation of carbohydrate composition, structural configuration, and mode of attending glycosidic bonds, molecular weight and other structural characteristics. Moreover, the monosaccharides in the EPS can be acted as reductive agents and these can add hydrogen, which when combined with radical forms a more stable radical to terminate the free radical chain reaction.

The model of scavenging the DPPH radical is a widely to evaluate the free radical-scavenging activities of polysaccharides **(Soares *et al.*, 1997; Naik *et al.*, 2003).** The method is based upon the decrease in the absorbance of methanolic solution of DPPH at 517 nm in the presence of EPS which acts as a proton-donor as shown in figure 1. EPS may be reacting with DPPH radicals to convert them to more stable products and thereby terminate radical chain reactions.

The superoxide radicals are generally formed first in the cellular oxidation reactions and its effect can be a precursor for the generation of other types of cell-damaging free radicals **(Liu and Ng, 2000).** The superoxide radical was generated by the autooxidation of pyrogallol and it can form a coloured compound resulting purple to yellow colour. The absorbance at 320 nm increased when the superoxide radical was scavenged by an antioxidant. The increment in the absorbance represent the content of superoxide anions and also indicate the antioxidant activity of the sample **(Chen *et al.*, 2008).** The results suggested that the EPS can be used as effective scavenger for superoxide radicals (figure 2), and it might be advantageous for preventing injury caused by superoxide radicals in adverse pathological conditions.

Hydroxyl radicals are known as highly potent oxidant, which can react with most of the biomacromolecules in living cells and induce severe catabolic activities to the adjacent biomolecules. Therefore, it is important for removing hydroxyl radical for antioxidant defence in cell or food systems. The present results proved that the EPS isolated from *Micrococcus luteus* SNIST- CM02 was a good scavenger for hydroxyl radicals as shown in figure 3.

Chelating agents may serve as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ions. It was concluded that the microbial EPS could be used as a novel chelating agent from this studies. The chelating ability was comparable with the EDTA as shown in figure 4.

The lipid peroxidation is a process, in which the free radicals steal the electron from the lipids of the cell membrane. This results in cell damage. This is generally occurred in various pathological events like inflammation and cellular aging **(Wiseman and Halliwell, 1996).** In this study, a yolk of egg suspension was used to evaluate the inhibitory activities of lipid peroxidation with EPS. The egg yolk lipids undergo rapid peroxidation when incubated with $FeCl_2$. The inhibiting effects of the EPS on the lipid peroxidation was found to be a concentration dependent process as shown in figure 5.

CONCLUSION

In this paper, we have studied the antioxidant aspect like scavenging of DPPH radicals, scavenging of super oxide radical, scavenging of hydroxyl radical, chelating of metal ion and lipid peroxidation inhibition studies of the EPS synthesized by *Micrococcus luteus* SNIST- CM02. The present investigation suggested that the EPS isolated from *Micrococcus luteus* SNIST-CM02 could be helpful and beneficial to alleviate the oxidative damages in cell induced by oxygen radicals and decelerate the progress of many chronic diseases in human body. However, the *in vivo* antioxidant activity and the antioxidant mechanism of EPS need to be analyzed in future.

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ANTIMICROBIAL ACTIVITY of *SPIRULINA PLATENSIS* AGAINST AQUATIC BACTERIAL ISOLATES

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ABSTRACT

Aquatic organisms are a rich source of novel and bioactive compounds. Cyanobacteria and microalgae being a rich source of bioactive compounds have recently found immense application in human and animal medicine. The present study was attempted to find out the effect of the various extracts of *Spirulina platensis*, *Chlorella vulgaris*, *Saragassum wightii* and *Saragassum latifolium* using different solvents (methanol, ethanol, ethyl acetate and chloroform) as antimicrobial agents against five bacterial pathogens; *S. aureus*, *E. coli*, *P. aeruginosa*, *Salmonella* sp, *Shigella* sp. Results indicate that among the various extracts used, methanol extracts of tested cyanobacterial and algal species appeared to be the most effective ones showing maximum antibacterial activity against the selected bacterial pathogens. *Spirulina platensis* appeared to be the most effective against all the pathogens studied. The antibacterial substance was purified using column chromatography. The nature of the purified active fractions was detected using different chemical analyses (UV, FT-IR, ^{1}H NMR and GC-MS) which indicated that it is an aliphatic compound and has different active groups (-OH,-C=O,-CH_2 and –CH_3). The results of this investigation proved that the tested cyanobacterium could be a good source for the production of promising antimicrobial agents.

Keywords: *Spirulina platensis*, Antimicrobial activity, H-NMR, FT-IR, GC-MS

INTRODUCTION

Bacterial infection causes high rate of mortality in human population and aquaculture organisms (**Kandhasamy and Arunachalam, 2008**). Preventing disease outbreaks or treating the disease with drugs or the screening programs for selecting therapeutic chemicals tackles this problem. The search for natural compounds with antimicrobial activity has gained importance in recent years due to growing worldwide concern about alarming increase in the rate of infection by antibiotic resistant microorganisms (**Kaushik and Chauhan, 2008**). Various strains of cyanobacteria and algae are known to produce intracellular and extracellular metabolites with diverse biological activities such as antialgal, antibacterial, antifungal and antiviral activities (**Noaman *et al.*, 2004; Kumar *et al.*, 2011; Al-Wathnani *et al.*, 2012**). Numerous substances were identified as antimicrobial agents from algae such as Chlorellin derivatives, acrylic acid, halogenated aliphatic compounds, terpenes, sulphur containing heterocylic compounds, phenolic inhibitors etc. (**Lavanya and Veerappan, 2011**). It is generally considered that compounds produced naturally, rather than synthetically, will be biodegraded more easily and will therefore be environmentally acceptable (**Ozdemir *et al.*, 2004; Colla *et al.*, 2007**).
Attention is now being focused on the natural components produced by aquatic organisms. Cyanobacteria are potential sources of high value chemicals and pharmaceuticals (**Tan, 2007**). The cyanobacteriaum, *Spirulina platensis* has emerged as one of the most promising agents to synthesize potentially new therapeutic compounds. It is known to produce intracellular and extracellular metabolites with diverse biological activities such as antifungal (**MacMillan *et al.*, 2002**), antiviral (**Hayashi and Hayashi, 1996**) and antibacterial activities (**Kaushik and Chauhan 2008; Kumar *et al.*, 2011**).
The aim of the study is to: (1) measure *in vitro* the antibacterial activity of different extracts of some cyanobacterial and algal extracts against different aquatic microbial isolates collected from surface water of Al-Bahr El-Pherony, Menoufia, Egypt and (2) characterize the structure of active compound using different methods including; H-NMR, FT-IR, UV and GC-MS analysis.

MATERIALS AND METHODS

Bacterial isolation

The microorganisms used in the antibacterial assay were isolated from surface water of Al-Bahr El-Pherony, Menoufia, Egypt. The obtained isolates were identified as previously explained by **Sabae *et al.* (2014)**. One Gram-positive bacterium namely *Staphylococcus aureus*, four Gram-negative bacteria namely *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* sp, *Shigella* sp. were tested for the antibacterial activity.

Inoculum preparation

The bacterial isolates were inoculated on Müller Hinton broth and incubated for 24 h at 30°C then suspended in saline solution 0.85% NaCl, adjusted to yield approximately 1.0 x 10^7-1.0 x 10^8 CFU/ml by using spectrophotometer (25% transmittance at 530 nm).

Cultivation of tested species

Spirulina platensis and *Chlorella vulgaris* were obtained from the culture collection of the Botany Department, Faculty of Science, Tanta University, Tanta, Egypt. Zarrouk`s medium (**Zarrouk, 1966**) was used for cultivation of *S. platensis* while, Kuhl`s medium was used for cultivation of *Chlorella vulgaris* (**Kuhl, 1962**). Culture temperature was maintained at 30 ±1°C. They were grown until the late exponential phase of the growth at which the cultures were harvested. The collected biomass was dried in a hot air oven at 60°C for 1 h.

Collection of macroalgae

Saragassum wightii and *Saragassum latifolium* samples were collected from the rocky areas surface in Red sea beach, Seuz, Egypt during spring 2013. After collection, samples were washed with seawater to remove epiphytes and other marine organisms. The seaweeds were transported to the laboratory in sterile

plastic bags. In the laboratory, samples were rinsed with tap water, identified following **Abbott and Hollenberg (1976); Aleem (1993) and Taylor (1985)** and dried on shadow at room temperature (25 to 30°C) Dried sample were cut into small pieces and powdered in a mixer grinder to get fine powder. Obtained powdered samples were stored in tight plastic bags.

Preparation of various extracts

Antibacterial extracts were prepared according to the method adopted by **Kaushik and Chanhan (2008)** by mixing 10 g of dried cyanobacterial and microalgae biomass to 150 ml of solvents (methanol, ethanol, chloroform and ethyl acetate) for 5 h at room temperature and sonicated for 15 min, for sea weeds the air dried samples were mixed with the respective solvent (1:15 w/v) for 72 h at room temperature with occasional shaking **(Osman *et al.*, 2013)** and then filtered through Whattman filter paper No.1. The obtained extract was freed from solvent by evaporation under reduced pressure and then resuspended in the appropriate solvent to make the solution of known concentration of 50 mg/ml. The extract was stored at 4°C in airtight glass bottle for the antibacterial assay.

Antibiotic susceptibility testing

The susceptibility of the recovered bacterial isolates to 24 different antibiotics representing 14 different classes was performed by modified Kirby-Bauer single-disk diffusion technique on Müller Hinton agar (**Robert *et al.*, 2003**). Ampicillin, oxicillin, carbenicillin, azteronam, ampicillin-sulbactam, pipracillion-tazobactam, cephalothin, cefatizidime, cefotixin, impenim, tobramycin, gentamicin, ciprofloxacin, chloramphenicol, tetracycline, erythromycin, rifampin, erythromycin, streptomycin, norfloxacin, ofloxacin, trimethoprim/sulfamethoxazole, nitrofurantoin and clindamycin were used for determination of antibiotic resistance profiles of the isolates. The results of the susceptibility tests were interpreted according to the criteria established by the Clinical and Laboratory Standards Institute (**CLSI, 2010**). Selected multidrug resistant bacteria were used in antibacterial assay.

Antibacterial assay

Antibacterial activity of the extracts was determined by microplate reader assay method according to **Bechert *et al.* (2000)** with some modifications. Aliquot of 100 µl of bacterial isolate (10^6 CFU/ml) in Müller Hinton broth medium was transferred to each well of 96 well plate. Volumes of 50 µl of extracts were added to each well in triplicate. The plates were incubated at 37°C for 24 h. After incubation, the absorbances of the plates were determined using automated ELIZA microplate reader adjusted at 620 nm. In every microtiter plate, one raw was set for positive control (A0) without tested extract against DMSO and fresh Müller Hinton broth medium as negative controls (A2). While, the used extracts mixed with fresh medium was used as a blank group (A1).The inhibition percentage of tested extracts was calculated according to the following equation (**Mulyono *et al.*, 2012**).

Inhibition percentage = $100 - \left(\frac{A - A1}{A2 - A0} \times 100\right)$ Where,

A : The absorbance of the treatment group

A1 : The absorbance of the blank.

A2 : The absorbance of the negative control group.

A0 : The absorbance of the positive control group.

Determination of the chemical structure of antagonistic material

Column chromatography

The methanol extract of one gram of *Spirulina platensis* was applied in a silica gel column (60-120 mesh), and eluted with a mixture of toluene and ethyl acetate (10:1 to 1:10). The collected fractions were freed from solvents by evaporation until complete dryness in a rotary evaporator. The dried samples were dissolved in pure methanol to a final concentration of 5% and assayed for their antibacterial activity against *P. aeruginosa* and *S. aureus* using agar well diffusion assay.

Ultra-Violet spectra (UV)

The UV-spectra of the tested material were determined using UV2101/pc spectrophotometer. The wavelength ranged from 200 to 800 nm.

Fourier transform-infrared spectroscopy (FT-IR) analysis

The active fractions were analyzed using FT-IR spectroscopy. The unutilized balance fraction samples were encapsulated in KBr at a ratio of 1:100. The IR spectra were collected using a Shimadzu spectrometer within the range of 500-5000 cm^{-1}.

Proton Nuclear Magnetic Resonance (^{1}H NMR) spectra

The sample was dissolved in deuterated chloroform. The different functional groups were identified using NMR (Varian Mercury VX-300).

Gas Chromatography -Mass Spectroscopy (GC-MS) analysis

A sample of the extracted fraction was subjected to GC-MS (Perkin Elmer) analysis. Phytoconstituents of the sample were analyzed using Perkin Elmer Clarus 580 series gas chromatographic system and capillary column. Rtx-5ms (5% phenyl, 95% dimethylpolysiloxane-Column length: 30m Column id: 250 µm) was used with helium at a 1.2 ml/min as the carrier gas and the GC oven temperature was programmed at 270-280°C. Identification of the individual components was performed by comparison of mass spectra fragmentation pattern with the profiles from the Wiley GC-MS 275 libraries.

Statistical analyses

The results are presented as mean ± standard deviation of the mean (n = 3). The statistical analyses were carried out using SPSS program version 15. Data obtained were analyzed statistically to determine the degree of significance between treatments using one and three way analysis of variance (ANOVA) at $P \leq 0.01$ and $P \leq 0.05$ levels of significance.

RESULTS

Screening for antibacterial activity

Different recovered bacterial isolates possessed multi-drug resistant (MDR) pattern to different used antibiotics Table (1). Hence, the MDR isolates were selected for the antibacterial assay against tested extracts. Antibacterial activities of crude extracts of the tested cyanobacterium and algae were determined by ELISA microtiter plate reader and the results are summarized in Table (2). The extracts showed varying degrees of antibacterial activity against all five pathogenic bacteria tested. On a general note, methanolic extracts exhibited higher degree of inhibitory activity than other used solvents. The stated results indicated that the most promising organism for the production of the antibacterial agent was *S. platensis* against all tested bacteria. Therefore, it was selected for further investigations.

Table 1 Percentage rates of resistance of different bacterial isolates to different antimicrobial agents.

Antimicrobial agent	No. (%) of resistant isolates				
	E. coli (n=7)	*Salmonella* sp. (n=7)	*Shigella* sp. (n=7)	*S. aureus* (n=7)	*P. aeruginosa* (n=7)
Ampicillin	6 (85.7 %)	7 (100 %)	6 (85.7 %)	-	-
Carbincillin	7 (100 %)	7 (100%)	4 (57.14 %)	-	7 (100 %)
Oxacillin	-	-	-	7 (100 %)	-
Amoxacillin	-	-	-	5 (71.4 %)	-
Azteronam	-	-	-	-	7 (100%)
Ampicillin/ Sulbactam	0 (0 %)	3 (42.86%)	1 (14.3 %)	0 (0 %)	-
Pipracillin/Tazobactam	-	-	-	-	0 (0 %)
Cephalothin	4 (57.1%)	4 (57.14%)	5(71.4 %)	6 (85.7 %)	-
Cefatizidime	7 (100%)	7 (100%)	7 (100%)	-	6 (85.7%)
Cefotixin	7 (100%)	7 (100%)	6 (85.7 %)	-	6 (85.7%)
Impenim	0 (0%)	2 (28.6%)	1 (14.3%)	0 (0 %)	0 (0%)

Gentamycin	0 (0%)	0 (0%)	0 (0%)	-	0 (0%)
Tobramycin	0 (0%)	2 (28.6%)	2 (28.6 %)	-	0 (0%)
Streptomycin	-	-	-	3 (42.7%)	-
Rifampin	-	-	-	6(85.7%)	-
Ciprofloxacin	1 (14.3%)	1 (14.3%)	2 (28.6 %)	0 (0 %)	1 (14.3%)
Ofloxacin	0 (0%)	0 (0%)	0 (0 %)	-	1 (14.3%)
Norfloxacin	0 (0%)	1 (14.3%)	2 (28.6 %)	-	0 (0%)
Co-trimoxazole	0 (0%)	2 (28.6 %)	2 (28.6 %)	2(28.6%)	3 (42.86%)
Clindamycin	-	-	-	7(100%)	-
Erythromycin	-	-	-	7(100%)	-
Nitrofurantoin	6 (85.7 %)	6 (85.7 %)	3 (42.86 %)	4(57.14%)	-
Chloroamphenicol	0 (0%)	3 (42.9 %)	5 (71.4%)	6(85.7%)	5 (71.4%)
Doxycycline	0 (0%)	0 (0%)	1 (14.3 %)	1(14.3%)	2 (28.6 %)

n: number of bacterial isolates

Table 2 The mean inhibition percentage of different cyanobacterial and algal extracts against bacterial isolates recovered during summer from study area.

Tested extracts	Bacterial isolates				
	E. coli	*Salmonella* sp.	*Shigella* sp.	*S. aureus*	*P. aeruginosa*
	Inhibition percentage (%)				
	Spirulina platensis				
Methanol	97.87±4.1	98.07±9.0	95.84±4.5	93.67±8.7	95.17±4.6
Ethanol	91.07±5.1	75.36±6.7	83.11±2.7	90.7±7.0	86.39± 6.3
Ethyl acetate	77.79±4.8	81.94±8.0	91.37±5.3	88.74±6.2	81.18±7.6
Chloroform	74.33±6.6	82.53±6.4	87.09±4.5	90.83±5.6	66.97±6.2
Chlorella vulgaris					
Methanol	92.27±6.8	92.61±5.0	90.29±5.2	85.79±7.4	82.01±5.3
Ethanol	87.51±7.5	90.64±5.1	86.43±7.2	81.96±5.5	71.89±6.4
Ethyl acetate	67.36±5.5	86.37±2.8	66.71±6.7	55.89±3.4	81.19±6.8
Chloroform	65.84±6.3	87.51±9.1	66.21±5.4	85.31±8.4	78.36±5.3
Saragassum wightii					
Methanol	89.38±5.6	84.93±6.5	84.56±4.5	92.61±8.6	88.03±4.7
Ethanol	81.43±6.5	87.1±5.1	74.13±5.7	67.7±5.5	83.57±6.3
Ethyl acetate	75.01±6.7	81.64±7.7	79.04±4.1	71.71±6.1	87.14±4.7
Chloroform	73.21±5.9	77.26±7.4	78.87±4.1	89.1±7.8	75±5.4
Saragassum latifolium					
Methanol	88.89±5.7	87.94±5.1	89.83±10.3	92.86±8.2	85.39±5.1
Ethanol	79.84±6.6	68.74±5.3	88.74±4.5	90.94±7.2	83.67±8.0
Ethyl acetate	81.44±7.6	86.99±7.3	83.04±6.5	83.56±4.4	70.57±6.3
Chloroform	73.91±5.8	67.31±6.1	81.51±27.1	84.57±8.1	84.1±5.2

Each value is the mean of three readings ± Standard Deviation Purification and characterization of the highly active crude extract of *Spirulina platensis*

Column chromatography

The methanol extract of *Spirulina platensis* was applied in a silica gel column and it was eluted with a mixture of toulene and ethyl acetate (10:1 to 1:10). The obtained 45 fractions were collected, tested for their antibacterial activity against *P. aeruginosa* and *S. aureus* using agar well diffusion. Only four fractions had antimicrobial activity as shown in Table (3). The UV absorption spectra of these fractions were determined sing spectrophotometer (UV 2101/ pc) at range of 200 to 800 nm. The obtained results are shown in Figure 1. The results indicated that the four fractions had the same absorption peaks (three absorption peaks at 285, 365 and 510 nm). Therefore, they were pooled together and subjected to various chemical analyses to reveal its structure as far as possible.

Table 3 The antimicrobial activities of the different fractions obtained from the silica gel column chromatography against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

No. of active fractions	Diameter of inhibition zone (mm)	
	S. aureus	*P. aeruginosa*
10	13±0.04	11±0.03
11	20±0.03	11.5±0.02
12	12±0.06	None
13	14±0.06	12±0.03

Total number of collected fractions= 45

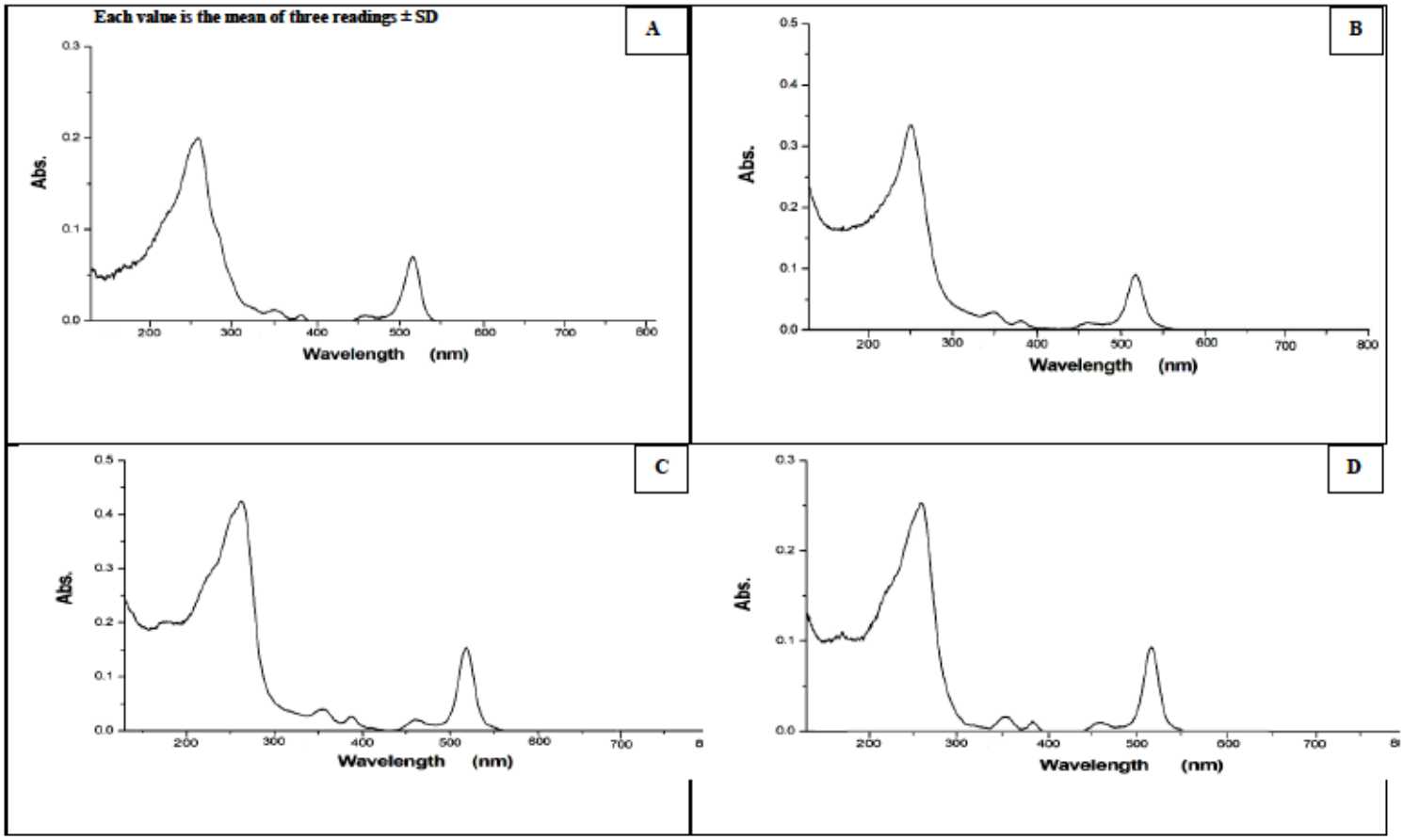

Figure 1 UV spectrophotometer scanning of different active fractions separated by column chromatography. A: fraction 10, B: fraction11, C: fraction12 and D: fraction13.

Chemical characterization of the purified active compounds

FT-IR spectroscopy

The FT-IR spectrum showed three absorption bands (Figure 2); the first band appeared at 3424 cm^{-1} due to OH group, the second band appeared at 2958 cm^{-1} referred to the C-H aliphatic and the third band appeared at 1729 cm^{-1} attributed to the carbonyl group (C=O).

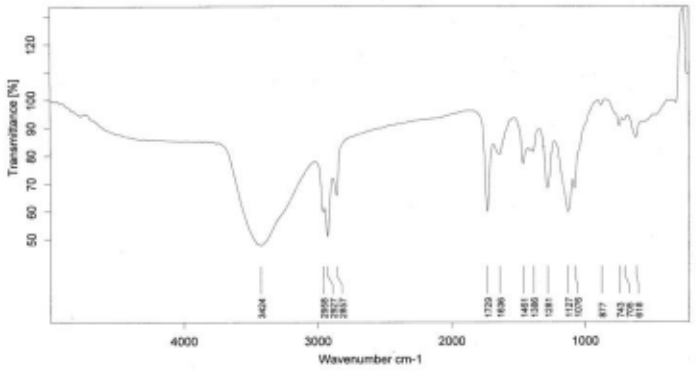

Figure 2 FT-IR spectrum of the purified antibacterial substance produced by *S. platensis*.

Nuclear magnetic resonance spectra

The ^1H NMR spectrum of the compound under investigation is measured in deturated chloroform ($CDCl_3$) as a solvent. The characteristic signals within the ^1H NMR spectrum was represented graphically in Figure 3. The proton NMR spectrum of the compound under investigation showed signals (ppm) at: δ0.9 (t, 3H, CH_3), δ 1.3 (t, 2H, CH_2), δ 2.3 (s, 2H, CH_2) and δ7.2 (COOH group).

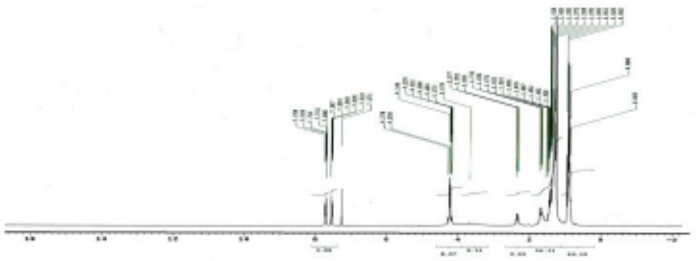

Figure 3 ^{1}HNMR spectrum of the antibacterial substance obtained from *S. platensis* methanolic

Gas Chromatography-Mass Spectroscopy (GC-MS) analysis

Based on the GC-MS results, thirteen bioactive compounds were identified from the partially purified fractions of the cyanobacterium *S. platensis*. The relative percentages of the compounds are given in Table (4). The most prevalent compound was n-Hexadecanoic acid (34.28%). Mass spectrum indicated that the molecular weight of the compound under investigation was 256 Dalton (Figure 4). Finally, on the basis of UV, IR, ^{1}H-NMR, and mass spectral data it was concluded that the compound was n- hexadecanoic acid with chemical formula: $C_{16}H_{32}O_2$.

Table 4 GC-MS analysis of different compounds in active fractions of *S. platensis* methanolic extract.

Peak	Rt (min)	Area (%)	Name
1	5.929	2.798	Butane, 1-ethoxy-
2	7.589	1.419	2,2-Dimethoxybutane
3	15.162	0.515	Oxalic acid, isobutyl pentyl ester
4	32.624	0.817	5-Isopropyl-6-methyl-hepta-3,5-dien-2-ol
5	33.056	1.438	2-Pentadecanone, 6,10,14-trimethyl-
6	33.905	0.961	Pentadecanoic acid, 14-methyl-, methyl ester
7	34.105	1.948	cis-9-Hexadecenoic acid
8	**34.275**	**30.604**	**n-Hexadecanoic acid**
9	34.590	1.993	Octanoic acid, 4-methyl-, ethyl ester, (ñ)-
10	35.861	1.030	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
11	36.051	1.179	Ethanol, 2-(9-octadecenyloxy)-, (Z)-
12	36.601	1.008	11,13-Dimethyl-12-tetradecen-1-ol acetate
13	38.807	0.684	1,2-Cinnolinedicarboxylic acid, 1,2,3,5,6,7,8,8aoctahydro-4-trimethylsilyloxy-, diethyl ester

RT: retention time

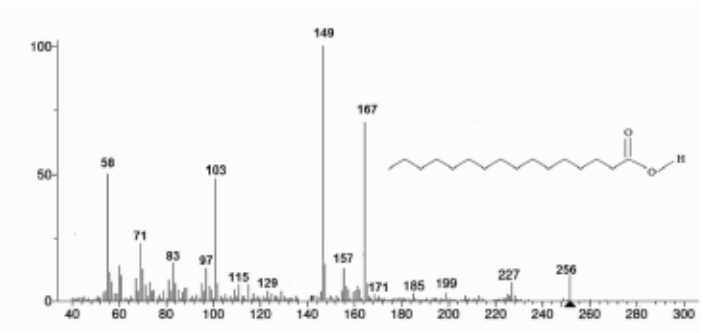

Figure 4 Mass spectrum of the antibacterial substance obtained from *S. platensis* (n-Hexadecanoic acid)

DISCUSSION

Al-Bahr El-Pheroany is an important watershed and a crucial source of irrigation water. It is considered one of the important sources of fisheries in Menoufia Government, Egypt. Plants and fishes living in these water bodies, when poisoned with harmful chemicals and metals can't survive (**Ghannam *et al.*, 2014**). The crops and vegetables irrigated with such polluted water become harmful for human beings. The level of pollution is swelling day by day due to non-availability of proper drainage system for industrial units and housing societies established along the banks. The ever-growing level of pollution in water is posing serious threats to human health besides making the water harmful for irrigation and fisheries purposes. Microbiological assessment of the water stream revealed the presence of rich communities of both indicator and human pathogenic bacteria (**Sabae *et al.*, 2014**).

Microorganisms have developed adaptation mechanisms against the action of antimicrobial drugs (**Al-Haj *et al.*, 2009**). This problem is one of the main reasons for continued research into antimicrobial compounds, including molecules from cyanobacteria and marine algae (**Kim *et al.*, 2007; Al-Wathnani *et al.*, 2012**). Much attention is being paid towards plant extracts and biologically active compounds isolated from natural resources in the present. Aquatic organisms are a rich source of structurally novel and biologically active metabolites (**Ely *et al.*, 2004**). Secondary or primary metabolites produced by these organisms may be potential bioactive compounds of interest in the pharmaceutical industry (**Prakash *et al.*, 2011**). Most of the secondary metabolites produced by seaweeds have bactericidal or the antimicrobial compounds derived from seaweeds consist of diverse groups of bacteriostatic properties terpenols, sterols, polysaccharides, dibutenolides peptides and proteins metabolites. Compounds with antibacterial activity have been detected in green, brown and red algae (**Yuan *et al.*, 2005; Bansemir *et al.*, 2006; Chew *et al.*, 2008**). Lipid soluble extracts from marine macroalgae have been investigated for their antibacterial properties.

In the present investigation, a high antibacterial activity of the studied cyanobacterium, namely; *Spirulina platensis* and algal species namely *Saragassum wightii*, *Saragassum latifolium* and *Chlorella vulgaris* have been reported against both Gram positive and Gram negative bacteria. These results were in accordance with the data obtained by several workers (**Sastry and Rao, 1995; Priya, 2012; Kumar *et al.*, 2011; Al-Wathnani *et al.*, 2012**).

Several different organic solvents have been used to screen algae for antibacterial activity (**Jeyanthi Rebecca *et al.*, 2012**). Similar to the results found in the current study, methanol seemed to be the best solvent for extracting the bioactive compounds.

In this study, the cyanobacterium *S. platensis* had the most effective antibacterial activity against both Gram positive and Gram negative bacteria compared with other screened algae and these results are in agreement with the findings by **Abdo *et al.* (2012)** and **Kaushik and Chauhan (2008)**. The collected fractions of the methanol extract of *S. platensis* using silica gel chromatography showed a high inhibitory activity against *S. aureus* and *P. aeruginosa*. Physical and chemical characterizations of the most active fractions were applied. From UV analysis, maximum absorption spectrum at 285 nm was observed. Accordingly, the composition of the active antimicrobial material was suggested to contain an aliphatic chain. The FT-IR spectroscopy indicated the presence of many functional groups: the first band appeared at 3424 cm^{-1} due to OH group, the second band appeared at 2958 cm^{-1} due to the C-H aliphatic and the third band appeared at 1729 cm^{-1} due to the carbonyl group (C=O). The ^{1}H NMR spectrum signals (ppm) at: δ0.9 (t, 3H, CH_3), δ1.3 (t, 2H, CH_2), δ 2.3 (s, 2H, CH_2) and δ7.2 (COOH group).

According to the results of UV, IR, ^{1}H-NMR and mass spectral data, it was concluded that the compound was n- hexadecanoic acid. The results obtained herein are supported by **Colla *et al.* (2007)** who reported major fatty acids extracted from the *S. platensis* as palmitic acid (C16:1), stearic acid (C18:1), oleic acid, linoleic acid etc. **Al-Wathnani *et al.* (2012)** observed that GC-MS analysis of the volatile components of *S. platensis* resulted in the identification of 15 compounds which constituted 96.45% of the total compounds. The volatile components of *S. platensis* consisted of heptadecane (39.70%) and tetradecane (34.61%) as major components.

Antimicrobially active lipids and active fatty acids are present in a high concentration in *Skeletonema costatum* (**Lampe *et al.*, 1998**). It was hypothesized that lipids kill microorganisms by leading to disruption of the cellular membrane e.g. bacteria, fungi and yeasts (**Bergsson, 2005**).They can also penetrate the extensive meshwork of peptidoglycan in the bacterial cell wall without visible changes and reach the bacterial membrane leading to its disintegration. This can probably be explained by the strong fabric of the cell wall of Gram-positive bacteria, which maintain their structure in spite of substantial hydrostatic turgor pressure within the bacteria (**Bergsson *et al.*, 2002; Shanmugapriya and Ramanathan, 2011**).

CONCLUSIONS

From the presented results it can be concluded that the extract of cyanobacterium, *S. platensis*, contains potential bioactive compound with an effective antibacterial activity. This compound can be utilized for the development of natural antibiotics against multi drug resistant bacteria.

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VIRULENCE STUDIES OF *PASTEURELLA MULTOCIDA* IN MICE, DUCKLINGS AND ADULT DUCKS

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ABSTRACT

Pasteurella multocida is a Gram negative bacterium causing severe disease in a multitude of hosts; the A: 1 strain of this bacterium is extremely virulent to hosts. In this study, the virulence of *P. multocida* A: 1 strain was assessed in one month old ducklings, six months old ducks and eight weeks old albino mice. The groups of experimental animals were inoculated with the different dilutions of the bacteria through subcutaneous route. The control groups were sham inoculated with sterile phosphate buffered saline via respective routes. Mortality was recorded for two weeks post inoculation. All the dead animals and birds were examined for specific gross lesions of pasteurellosis and attempted re-isolation of the organism on blood agar from their internal organs. The median lethal dose was calculated using Reed and Muench method. For ducklings, the median lethal dose was 13 colony forming units and the dilution giving 50 % end point with 0.1 ml subcutaneous dose was $10^{-7.4}$. The median lethal dose could not be arrived at for mice and adult ducks as the strain killed all the inoculated mice even in the lowest dilution tested, while most of the adult ducks were resistant to infection.

Keywords: *Pasteurella multocida,* Lethal dose, Virulence, Duckling, Mice, Gross pathology, Lesion score

INTRODUCTION

Pasteurella multocida (P. multocida), the Gram negative bacterium is responsible for numerous economically significant diseases in a variety of hosts like cattle, buffalo, sheep, goat, domestic fowl, turkey, horse, camel, wild animals and wild birds (**Snipes *et al.*, 1988; Boyce *et al.*, 2004**). Pasteurellosis is a persistent problem in developing countries especially in South East Asia and Africa. *P. multocida* have many serotypes, of which Serotype A is mainly responsible for causing Fowl Cholera or Avian pasteurellosis.

Several virulence studies had been conducted by many researchers to quantify the virulence of *Pasteurella* through various routes (**Rimler and Glisson, 1997**). All the studies echo one thing that the virulence of bacteria is complex, multi-factorial and it can vary depending on the strain used, host studied, route, dose of administration and many other unknown confounding variables. Virulence of a pathogen is measured in suitable experimental animals, the end result of which is death. Virulence testing forms an integral part of vaccinology and pharmacology, especially for computing the challenge dose during potency testing of vaccines and various drugs. Fifty per cent lethal dose or LD_{50} is defined as the number of bacteria required to kill half of the exposed hosts and is shown to be a practical and reliable measure of pathogenicity (**Thomas and Elkinton, 2004**).

The study was done to assess the virulence of avian origin *Pasteurella multocida* A: 1 strain in three different animal host models (1) one month old ducklings (2) six months old ducks and (3) eight weeks old albino mice.

MATERIALS AND METHODS

Bacterial strain

The *P. multocida* A: 1 strain was isolated from a very severe outbreak of pasteurellosis at Niranam Duck farm, Pathanamthitta District, Kerala State, India. The isolate was biochemically identified and serotyped as *P. multocida* serotype A, biovar 1 at Indian Veterinary Research Institute (IVRI), Izatnagar, India. The isolate was named as DP1 and maintained in freeze dried form at the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences (COVAS), Mannuthy, Kerala, India.

Experimental animals

Unvaccinated one month old ducklings (n=54) (*Anas platyrhynchos,* Kuttanad variety) and 6 months old Kuttanad ducks (n=54) were procured from a private breeder at Thrissur, Kerala, India. Swiss albino female mice (*Mus musculus*), eight weeks of age (n=54) were procured from the Small Animal Breeding Station, COVAS, Mannuthy. The ducklings were randomly assigned into nine groups, with six ducklings in each group. The ducks and mice were also similarly arranged into nine groups. The first eight groups served as the test groups while the ninth group served as control group, in all the three trials. Each group of birds was housed separately in locally made isolator cages (of dimensions 6×3×3 cubic feet) which were arranged in two adjacent rooms (4 cages in each room). The control birds were kept in a separate room in order to avoid any chances of cross contamination. Each cage had separate waterers and feeders and the floor being covered with saw dust as litter material. The birds were provided with commercial duck feed (formulated at University Poultry Farm, COVAS) and water *ad libitum*. The groups of mice were kept in plastic shoe box cages and provided with commercial mice feed (formulated at Small Animal Breeding Station, COVAS) and water *ad libitum*. The animals were housed in their respective cages one week prior to the beginning of the experiment for acclimatization. The animals and birds were observed for signs of any disease before the start of experiment.

All the animal experiments were performed with the prior approval of the Institutional Animal Ethics Committee (IAEC) of COVAS, Mannuthy, which follows the guidelines laid by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India.

Median lethal dose (LD_{50}) testing

For determination of LD_{50}, the freeze dried *P. multocida* A: 1 was reconstituted in 0.1 ml Tryptone soya broth (Himedia, Mumbai, India) and cultured on to dextrose starch agar (DSA) (Himedia, Mumbai, India) at 37°C for 24 h. The growth on DSA was harvested, washed thrice in phosphate buffered saline (PBS, pH 7.4) by centrifugation at 3000 × g for 15 min and re-suspended in the same buffer to contain 3×10^9 CFU ml^{-1} using McFarland standards (600 nm). Then

serial tenfold dilutions were made which were quantitatively assessed by plate counting retrospectively.

The test groups of ducklings were inoculated with the prepared dilutions of the bacteria at the rate of 0.1 ml per bird subcutaneously at wing web region. The adult ducks were also inoculated via wing web route but with an increased dose of 0.5 ml. The first eight groups of mice were inoculated with the dilutions, 0.1 ml per mice, intraperitoneally. The control groups were sham inoculated with corresponding volumes of sterile PBS (pH 7.4) via respective routes. Mortality was recorded up to two weeks post inoculation (PI).

All the dead birds and animals were examined for specific gross lesions caused by *P. multocida* and attempted re-isolation of the organism on blood agar from heart blood, liver and spleen under 5-10 % carbon dioxide tension. The live birds and animals after 14 days PI were euthanized and bacteriologically examined. Blood smears and organ impression smears (spleen and liver) were also examined following Leishmans staining.

The method described by **Reed and Muench (1938)** was used for LD_{50} calculation from the recorded cumulative mortality rates. Median lethal dose was calculated from the proportionate distance (PD) using the formula listed below.

The calculation of median lethal dose from PD is as follows,

$$PD = \frac{\text{(\% mortality at dilution next above 50 \%) - 50 \%}}{\text{(\% mortality at dilution next above 50 \%) - (\% mortality at dilution next below 50 \%)}}$$

$$\text{Log } LD_{50} = \text{(logarithm of dilution next above 50 \% mortality + (PD} \times \text{log dilution factor)}$$

Lesion scoring

To assess the lesions, a comprehensive post mortem examination was performed on each bird and the lesions were scored (0-3) for six organs including intestine, lungs, pericardium, heart, liver and spleen as per the criteria listed in Table 1. The same personnel scored the lesions of all dead birds to bring down the error while judging the lesions and care was taken to do the scoring under sunlight. Statistical analyses for lesion scores were conducted using one way Analysis of Variance. Duncan's multiple range test was used to compare means (Duncan, 1955).

Table 1 Scoring system for lesions induced by *P. multocida* in different organs

Organ	Score	Description of lesion
Intestine	0	No lesions
	1	Catarrhal enteritis
	2	Moderate enteritis with petechial haemorrhage
	3	Extensive serosal and mucosal haemorrhage
Lung	0	No lesions
	1	Congestion of lungs
	2	Pulmonary oedema
	3	Pneumonic lungs
Pericardium	0	No lesions
	1	Pericardial effusion
	2	Translucent pericardium, moderate pericarditis
	3	Opaque pericardium, severe pericarditis
Liver	0	No lesions
	1	Congestion of liver
	2	Pin point necrosis, hepatomegaly
	3	Extensively necrosed and pale liver
Spleen	0	No lesions
	1	Congestion of spleen
	2	Mild necrosis and spleenomegaly
	3	Mottling /haemorrahge in spleen
Heart	0	No lesions
	1	Hypertrophy of heart/slight haemorrhage
	2	Moderate haemorrhage on epicardium
	3	Severe epicardial and endocardial haemorrhages

RESULTS

Median lethal dose in ducklings

The dilution giving 50 % end point with 0.1 ml subcutaneous dose was $10^{-7.36}$. The LD_{50} of the isolate was 13 colony forming units (CFU) when tested in one month old ducklings. The percentage of animals dead and alive in each group of ducklings is furnished in Table 2 and the detailed calculations are presented in legend section of Table 2.

The gross lesions observed in experimentally infected ducklings were haemorrhages on epicardium, serous yellow fluid in pericardium (Fig. 1A) pin point and extensive necrosis of liver (Fig. 1B), pin point and echymotic haemorrhages in intestinal serosa and mucosa (Fig. 1C), necrosis and petechial haemorrhage in spleen and pulmonary oedema. The gross pathological lesions observed in different groups of inoculated ducklings and the lesion scores are presented in Table 3. Bacteriological examination revealed bipolar organisms from blood and organ impression smears and colonies suggestive of *P. multocida* from internal organs of all the succumbed ducklings. The control birds did not reveal any bacterial growth following organ culture.

Table 2 Median lethal dose of DP1 in one month old ducklings

Group	CFU per 0.1 ml inoculum	No: of birds tested	No: died	No: alive	Cumulative*			Mortality rate	% mortality
					Dead	Alive	Total		
1	2.9×10^{7}	6	6	0	41	0	41	41/41	100
2	2.9×10^{6}	6	6	0	35	0	35	35/35	100
3	2.9×10^{5}	6	6	0	29	0	29	29/29	100
4	2.9×10^{4}	6	6	0	23	0	23	23/23	100
5	2.9×10^{3}	6	6	0	17	0	17	17/17	100
6	2.9×10^{2}	6	6	0	11	0	11	11/11	100
7	2.9×10^{1}	6	4	2	5	2	7	5/7	71.4
8	2.9×10^{0}	6	1	5	1	7	8	1/8	12.5

Legend: *Cumulative total value for dead and live birds was obtained by adding in the direction of lowest to the highest values (shown by solid arrows).

Table 3 Lesion score of the ducklings post inoculation with DP1

Group	Intestine**	Lungs**	Pericardium**	Heart **	Liver**	Spleen*
1	2.67 ± 0.82^{a}	1.83 ± 0.41^{a}	1.33 ± 0.52^{ab}	3.00 ± 0.00^{a}	2.17 ± 0.75^{a}	0.50 ± 0.55^{a}
2	2.33 ± 0.52^{a}	1.50 ± 0.55^{ab}	0.50 ± 0.55^{cd}	2.83 ± 0.41^{ab}	3.00 ± 0.00^{b}	1.50 ± 1.38^{ab}
3	2.67 ± 0.52^{a}	1.17 ± 0.75^{abc}	1.83 ± 0.41^{a}	2.50 ± 0.55^{ab}	2.00 ± 0.89^{a}	2.00 ± 1.10^{b}
4	2.17 ± 0.41^{a}	1.33 ± 0.52^{ab}	0.67 ± 0.52^{bcd}	2.83 ± 0.41^{ab}	2.17 ± 0.41^{a}	0.83 ± 0.41^{a}
5	2.83 ± 0.41^{a}	1.00 ± 0.63^{bcd}	1.17 ± 0.98^{abc}	2.33 ± 0.52^{b}	1.67 ± 0.82^{a}	0.50 ± 0.55^{a}
6	2.83 ± 0.41^{a}	0.33 ± 0.52^{d}	0.33 ± 0.52^{d}	1.17 ± 0.41^{c}	3.00 ± 0.00^{b}	1.00 ± 0.63^{ab}
7	1.50 ± 0.58^{b}	0.50 ± 0.58^{cd}	0.25 ± 0.50^{d}	1.50 ± 0.58^{c}	1.50 ± 0.58^{a}	0.50 ± 0.58^{a}

Legend: Values expressed as mean ± standard deviation (for groups 1-6, n=6 and for group 7, n=4)
Letters with different superscript within a column differ significantly. ** ($P \leq 0.01$) and *($P \leq 0.05$).

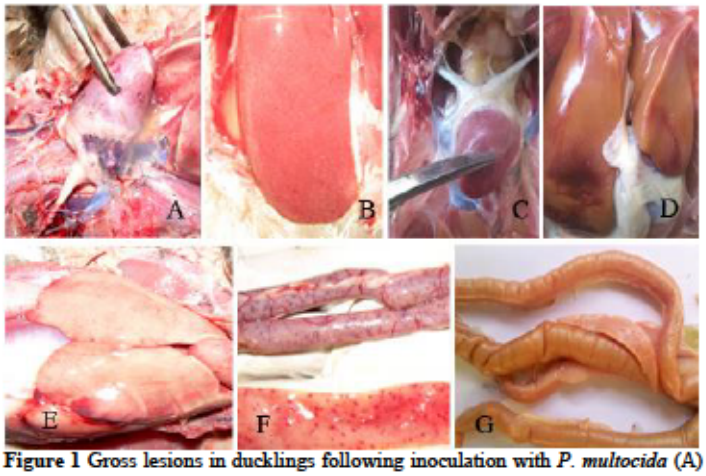

Figure 1 Gross lesions in ducklings following inoculation with *P. multocida* (A) Epicardial petechiae and serous pericardial effusion (lesion score 1) (B) Liver of duckling showing pin point necrosis (diffuse white spots) (lesion score 2) (C) Normal heart from a control bird (D) Liver from control bird showing no gross lesions (E) Extensively necrosed and pale liver (lesion score 3) (F) Multiple haemorrhagic spots in intestinal serosa and mucosa of dead ducklings (lesion score 3) (G) Intestine of control bird without any gross lesion.

Lethal dose 50 in adult ducks

Only one duck died, which was inoculated with 3×10^8 CFU/ml subcutaneously. The other ducks did not succumb and hence it was not possible to arrive at the median lethal dose. The dead duck revealed similar gross lesions as those observed in the case of ducklings. Additionally, extensive petechiation of the peritoneum was noticed (Fig. 2). Despite the successful isolation of the organism from the dead duck, none of the live birds from test groups revealed any of the gross lesions. No growth was observed for live birds on the media following organ culture. The inoculated birds showed some initial symptoms of dullness and drooping which subsided after one day post-inoculation.

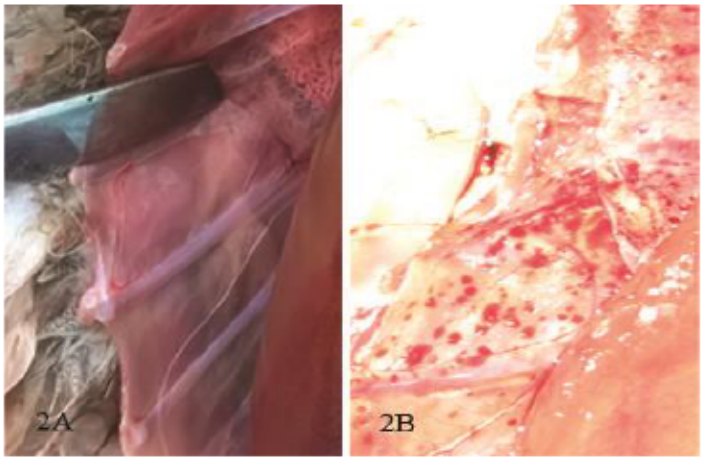

Figure 2 Extensive necrosis of liver and petechiae in peritoneal cavity of ducks experimentally infected with *P. multocida* (2B) compared to no gross lesions in control birds (2A).

Lethal dose in mice

Pasteurella multocida A: 1 strain killed all the inoculated mice within 2 days, even in lower dilutions, while all the mice in the control group remained unaffected. Because of the extreme pathogenicity of the bacteria in mice, the median lethal dose could not be obtained. The gross lesions observed in the inoculated mice were fluid accumulation in peritoneal cavity, petechial haemorrhages in the epicardium, pulmonary oedema and general congestion of all the visceral organs. It was interesting to note that the mice consistently showed peritoneal effusions unlike ducklings and the severe intestinal lesions shown by ducklings were not consistent in mice. Bipolar organisms were detected from blood smears, organ impression smears and the organism was successfully isolated from the internal organs of dead mice. The euthanized control mice revealed no gross lesions and no organisms isolated.

DISCUSSION

Pasteurella multocida possess a multitude of specific virulence factors like hyaluronic acid capsule, lipopolysaccharides, iron regulated outer membrane proteins (IROMPs) etc. This makes the host-bacterium interaction very complex and the virulence depends on the bacterial strain being studied, host model used and many other events occurring *in vivo*, most of which are yet to be elucidated. The gross lesions observed in ducklings were in agreement with **Mbuthia *et al.* (2008)** and **Shilpa *et al.* (2005)**.

Collins (1973) opined that an overwhelming increase in the number of organisms in visceral organs was the cause of death of mice when experimentally inoculated. **Ramdani *et al.* (1990)** observed that on injecting a *P. multocida* type B strain into BALB/c mice, as few as 20 CFU produced an overwhelming septicaemia in mice in less than 30 h, thereby revealing a very rapid *in vivo* multiplication rate. The *P. multocida* A: 1 strain used in this study killed the mice with less than 3 CFU and consequently we could not arrive at a median lethal dose, depicting a very high pathogenicity. Sotoodehnia *et al*. (**Sotoodehnia *et al.*, 2004**) also reported similar results with inability to arrive at median lethal dose because of very high virulence of avian *P. multocida* in mice. The post mortem lesions exhibited by dead mice were in conjunction with those reported by **Antony *et al.* (2007)**. They also observed that the *Pasteurella* strains isolated from ducks in Kerala which did not even posses any virulence plasmid, killed mice. **Ramanatha (1994)** determined LD_{50} of *P. multocida* A: 1 in mice and obtained a mean value of 14.32 + 0.083 CFU.

The mean lesion score for intestine was similar for all the groups except the seventh group for which it was lower. The lesion score was inconsistent for pericardium and spleen. Heart lesions were more severe in the groups inoculated with more number of organisms. Liver was equally affected in both lower and higher dilutions. The lung lesion score was reduced for groups inoculated with less concentrated inoculums. It was interesting to note that lung lesions induced by our strain were not that severe compared to other organs and this is unusual as *P. multocida* is regarded primarily as a respiratory pathogen. We could draw a correlation between the number of organisms in the inoculum and the lesion severity for organs like heart, intestine and liver.

Colonization and disease causation in a particular host tends to be associated with how well the bacteria get adapted to the host and its ability to overwhelm the host defenses. Resistance to infections is dependent upon a complex equilibrium between many constitutive as well as adaptive defense mechanisms which may be different for each host or even anatomical site of infection and for different pathogens (**Shilo, 1959**). **Hunter and Wobeser (1980)** demonstrated that mallard ducks older than 11 weeks are less vulnerable to *P. multocida* infection than younger ones. Similar age susceptibility was reported in family ducks by **Mbuthia *et al.* (2008)** and opined that more number of clinical signs with increased severity were shown by 4 and 8 weeks old ducklings than the older age groups. This clearly indicates that birds above 4 months have considerable resistance to pasteurellosis and lower doses of bacteria will be cleared by the host immune system. *P. multocida* loses its virulence on storage and multiple sub-culturing. The virulence can be enhanced by an *in vivo* passaging in a living host like mice. In the present study, in vivo passaging was done prior to inoculation to ducklings but not before inoculation to ducks. It may have influenced the outcome of the study, but it is unsure whether that alone contributed to the large variation in virulence in one month and six month old ducks. According to **Matsumoto and Strain (1993)**, *P. multocida* serotypes were able to increase their pathogenicity by bird to bird transmission. In their study, the encapsulated original isolate revealed a mean infectious dose of more than $10^{8.2}$ CFU which after five passages produced 67 per cent mortality with a 10^2 CFU dose. In the present study, only one duck died even in the highest dose of challenge. From this it is obvious that Kuttanad ducks are fairly resistant to pasteurellosis as age advances and they plausibly harbour some disease resistant genes which provide inherent immunity to diseases prevalent in Kuttanad, Kerala. Perusal of available literature did not reveal any genetic studies on disease resistance of Kuttanad ducks or resistance of ducks in general to pasteurellosis and so it warrants extensive studies on their genetic profile and disease resistance to pasteurellosis. The only adult duck that died PI revealed similar gross lesions as that of ducklings but additionally revealed extensive petechiation of mucous membranes of abdominal cavity.

CONCLUSIONS

The median lethal dose of *P. multocida* A:1 isolated from Niranam was 13 CFU in one month old ducklings. Due to inherent disease resistance and extreme lethality of the bacteria, it was not possible to arrive at a medial lethal dose in adult ducks and mice respectively. The dead birds and mice revealed all the classical lesions of pasteurellosis on post mortem examination and organism could be re-isolated from the visceral organs. The animal model should be carefully selected while performing lethal dose studies for it will hugely and single handedly influence the outcome as evidenced in this study. Mouse is not a suitable model for highly virulent strains of *P. multocida* and if used can lead to futile wastage of money, resources and animal lives. The age of the animal host should be optimally selected and correctly judged as it can also act as a critical determinant in obtaining good results.

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MICROWAVE MUTAGENESIS OF *BREVIBACILLUS PARABREVIS* FOR ENHANCED CELLULASE PRODUCTION, AND INVESTIGATION ON THERMOSTABILITY OF THIS CELLULASE

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ABSTRACT

Microwave mutagenesis of *Brevibacillus parabrevis* for enhanced cellulase production was attempted. Though microwave treatment could alter the cellulase activity of the test bacterium, none of the mutants obtained were found to be genetically stable, indicating the reversible nature of microwave-induced mutation(s). Thermal stability of the *B. parabrevis* cellulase was also investigated. This enzyme was found to be capable of retaining its activity even after heat treatment (50-121°C, for 30-60 min). Fluorescence spectrum revealed a *red shift* in the emission maxima of the heat-treated enzyme preparations, indicating some structural change upon heating, but no major loss of activity was observed. This enzyme was found to be active over a broad temperature range, with 90°C as the optimum temp, which is interesting as the producing organism is a mesophile.

Keywords: Cellulase, Microwave mutagenesis, Thermostability, Fluorescence spectroscopy

INTRODUCTION

The part of electromagnetic radiation corresponding to the frequency range of 300 MHz – 300 GHz is known as the microwave (MW) region. Thermal effect of the MW radiation is well established. However, there has been a heated controversy regarding the possible athermal (MW specific electromagnetic effects) effects of the MW radiation (**Trivedi *et al.*, 2011**). Literature contains reports indicating presence (**Porcelli *et al.*,** 1997) as well as absence (**Sasaki *et al.*, 1995**) of the athermal effects of the MW radiation. Few reports (**Gosai *et al.*, 2014**) have also appeared describing the mutagenic potential of MW radiation. *Brevibacillus parabrevis* is a gram-variable, aerobic, rod shaped (**Logan and Vos, 2009**) bacterium known for its cellulolytic activity (**Singh and Bansal, 2013**). Cellulases are enzymes of high industrial significance, particularly thermostable cellulases are of special interest. Either the cellulolytic thermophilic microbes or the purified thermostable cellulases can be of great use in alcoholic fermentation of lignocellulosic wastes (**Van Maris *et al*, 2006**). The present study aimed at obtaining a cellulase overproducing mutant of *B. parabrevis* using MW radiation as mutagenic agent. Besides this thermostability of the cellulase produced by the wild type *B. parabrevis* was also investigated.

MATERIALS AND METHODS

Test Organism

Brevibacillus parabrevis (MTCC 2708) was procured from Microbial Type Culture Collection (MTCC) Chandigarh.

Microwave Treatment

Bacterial suspension was prepared in sterile normal saline, from an active culture growing on nutrient agar (HiMedia, Mumbai). Inoculum density was adjusted to that of 0.5 McFarland standard i.e., 0.08-0.10 at 625 nm. Test culture (5 mL) in sterile screw capped glass vials (15 mL, Merck) was exposed to MW radiation (90 W; 2450 MHz) in a domestic MW apparatus (Electrolux® EM30EC90SS). MW treatment at 90 W was given for three different time durations viz. 2, 4, and 6 min. Vials inside the MW apparatus were placed in an ice-containing beaker, so as to avoid/minimize any thermal heating. Temperature of the microbial suspension after MW treatment at 90 W did not go beyond 14°C; when 90 W treatment was given for 6 min, temperature of the treated microbial suspension was found to be 13.3 ± 0.5°C. The whole MW treatment was performed in an air-conditioned room. Untreated inoculum was used as control. Before MW treatment all, the inoculum vials (including control) were put in ice for 5 min to nullify any variations in initial temperature. Test organism was immediately (in less than 5 min) inoculated onto the medium for screening of cellulolytic potential (**Gupta *et al*, 2012**), following MW treatment. This growth medium contained 0.5 g/L KH_2PO_4 (Merck, Mumbai), 0.25 g/L $MgSO_4.7H_2O$ (Merck), 0.2 g/L congo red (S.d. fine-chemi, Mumbai), 2 g/L cellulose (S.d. fine-chemi), 2 g/L gelatin,15 g/L agar-agar (HiMedia, Mumbai), pH 6.8-7.2.Incubation was made at 35°C for 24 h.

Estimation of cellulase activity

B. parabrevis was grown in a CMC (carboxymethyl cellulose; Merck) supplemented broth (Peptone 0.5 g/L; NaCl 0.5 g/L; Beef extract 0.5 g/L; Yeast extract 0.5 g/L; CMC-Na 20 g/L). Incubation of the experimental tubes was carried out at 35°C for 65 h, under shaking condition (80 rpm). The cell free supernatant obtained after centrifugation of the culture broth (10,000 rpm; 9,390 g for 15 min) was used as crude cellulase preparation. 0.5 mL of the supernatant was mixed with 0.5 mL of 1% CMC, followed by incubation at 50°C for 30 min. The amount of glucose released as a result of cellulase activity was quantified using DNSA colorimetric assay. The international units (IU) of the cellulase was calculated as: IU= (µg of glucose)/ 180 x 30 x 0.5 (**Nigam and Ayyagari, 2008**).

Screening for mutants

Following the MW treatment of *B. parabrevis* suspension, the treated inoculum was streaked on the screening medium plate (150 mm) containing congo red, and incubated at 35°C for 42-45 h. After the incubation 3 colonies from each plate corresponding to different MW treatments were picked (selection of the colonies was made based on the diameter of the zone of cellulose hydrolysis surrounding the colony), and each colony (a separate code was given to each picked colony) was streaked on to a separate nutrient agar (supplemented with 0.5% CMC) plate. Daughter populations thus generated (after 42-45 h incubation at 35°C) from a single parent colony were then inoculated into the liquid medium described in the preceding section for estimation of enzyme activity, followed by incubation at 35°C for 65 h under shaking condition (80 rpm). After incubation, cellulase activity was estimated for all the MW treated inoculums. Then the plates corresponding to the MW treatment yielding higher cellulase activity were selected for further experiments. Subculturing was done from the plates of cellulase overproducing mutant(s), and daughter population resulting from each subculturing was checked for its cellulase activity (in comparison to the wild type), up to 5 generations.

Partial purification of the enzyme preparation

B. parabrevis was inoculated into CMC containing broth, in 500 mL flasks. Volume of the growth medium in the flask was kept 250 mL. Incubation was carried out at 35°C under shaking condition (80 rpm) for 65 h. Cell free supernatant obtained after centrifugation of the *B. parabrevis* culture broth was subjected to ammonium sulphate (CDH, New Delhi) precipitation, wherein ammonium sulphate was added at the rate of 690 g/L. While adding ammonium sulphate [$(NH_4)_2SO_4$], the vessel containing the supernatant was kept surrounded by ice, and following that a 20 min incubation inside refrigerator was carried out. This was followed by centrifugation at 7,500 rpm for 45 min at 4°C. The resulting pellet containing the precipitated protein content was dissolved in 25 mM phosphate buffer. This solution was subjected to dialysis (11 KD pore size, 33 mm wide; Sigma Aldrich), wherein the dialysis bag was kept suspended in 25mM phosphate buffer for 12 h under refrigeration. At the end of dialysis the contain inside bag was centrifuged at 10,000 rpm (15 min; 4°C), and the resulting supernatant was used as partially purified cellulase preparation.

Finding the effect of temperature on enzyme-substrate interaction

0.5 mL of the partially purified enzyme was mixed with 0.5 mL of 1% CMC, followed by incubation for 30 min at different temp in the range 40-100°C. The amount of glucose released as a result of cellulase activity was quantified using DNSA colorimetric assay, and from that the international units (IU) of the cellulase was calculated. Appropriate controls containing only substrate (with no enzyme), and only enzyme (with no substrate) were also included in the experiment.

Investigating thermostability of the enzyme

The partially purified enzyme was subjected to heating in water bath for 1 h at different temp viz. 50°C, 80°C, and 100°C. Besides this it was also subjected to autoclaving for 30 min. One batch of these heated enzyme preparations was immediately subjected to estimation of cellulase activity. Another batch from the same lot was allowed to cool and undergo renaturation for 2 h. Following renaturation this was also subjected to estimation of cellulase activity. Additionally the renatured sample was also subjected to flurometric analysis to find out whether the reantured sample has undergone any structural change as compared to the unheated control. Fluorescence spectroscopy experiments were performed using cary eclipse fluorescence spectrophotometer, Agilent technology (US). Excitation wavelength used was 280 nm, and the emission was recorded in the range 300-400 nm.

Statistical analysis

All the experiments were performed in triplicate, and measurements are reported as mean ± standard deviation (SD). Statistical significance of the data was evaluated by applying *t*-test using Microsoft Excel®. *P* values less than 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Microwave mutagenesis

Following MW treatment of the *B. parabrevis* inoculum, it was streaked on to congo red containing screening medium, and the colonies surrounded by zone of cellulose hydrolysis bigger than that of control (Table 1) were selected for further experimentation. Each of the nine colonies shown in Table 1 was streaked on to a separate nutrient agar (supplemented with 0.5% CMC) plate. Daughter populations thus generated (after 24 h incubation at 35°C) from a single parent colony were then inoculated into the liquid medium described in the section for estimation of enzyme activity, followed by incubation at 35°C for 65 h under shaking condition (80 rpm). After incubation, cellulase activity was estimated for all the MW treated inoculums (Table 2). Then the plates corresponding to the MW treatment yielding higher cellulase activity were selected for further experiments. Subculturing was done from the plates of cellulase overproducing mutants (i.e. 4A, 6A, 6B, and 6C), and daughter population resulting from each subculturing was checked for its cellulase activity (in comparison to the wild type), till any significant higher cellulase activity was observed (Table 3). Out of the four mutant strain selected, none could maintain the trait of cellulase overproduction after third subculturing. Still we continued the experiments till fifth subculturing, and found all the mutants to revert back to the parent phenotype with respect to cellulase activity.

Though the MW radiation could exert its mutagenic effect on *B. parabrevis* cellulase activity, the mutants obtained were not found to be genetically stable. It can be said that the MW induced mutations in the cellulase synthesizing/secretion machinery observed in the study were of reversible nature. Literature contains reports indicating the MW induced mutations to be stable, as well as those indicating otherwise. **Pasiuga *et al.* (2007)** reported disappearance of low-level MW induced effects after few generations in *Drosophilla malenogaster*. **Kothari *et al.* (2014)** also showed mutagenic effect of MW radiation on exopolysaccharide production in *Xanthomonas campestris* to be of reversible type.

Whatever alterations in growth and cellulase activity of MW exposed *B. parabrevis* were observed in the study, seem largely owing to the non-thermal effects of MW radiation, as MW treatment in the study was provided at relatively low power (90 W) and the temperature of the MW treated suspension did not go beyond 14°C. Much cannot be said with certainty regarding the mode of action of MW radiation on living systems. **Bollet *et al.* (1991)** reported alteration in the cell membrane permeability owing to MW treatment, which in part may contribute to the non-thermal effects of MW on microbial cells. Besides thermal and non-thermal effects, micro-thermal effects incorporating 'undetectable' thermal mechanism may also be responsible for the biological effects of microwave radiation (**Shamis *et al.*, 2012**).

Table 1 Results of qualitative screening of the cellulolytic potential on congo red medium

Duration of MW treatment at 90 W (min)	Colony Code	Diameter of Zone of hydrolysis (mm) (Mean ± SD)	% increase compared to control
0 (Control)		8±1	0.0
2	2A	15	87.5
	2B	13	62.5
	2C	15	87.5
4	4A	11	37.5
	4B	10	25
	4C	10	25
6	6A	10	25
	6B	9	12.5
	6C	10	25

Table 2 Quantification of cellulase activity of the population generated from selected colonies

Colony Code	Cell density at harvest (OD_{660}) (Mean ± SD)	% Change compared to control	Cellulase activity (IU/mL)	% Change compared to control
Control	0.716±0.04	0.00	0.020	0.00
2A	0.613±0.06	-14.38	0.017	-15
2B	0.614±0.01	-14.24	0.018	-10
2C	0.623±0.01	-12.98*	0.023	15
4A	0.729±0.05	1.81	0.050	150**
4B	0.577±0.01	-19.41*	0.021	5
4C	0.695±0.00	-2.93	0.023	15
6A	0.749±0.06	4.60	0.039	95**
6B	0.737±0.02	2.93	0.047	135**
6C	0.693±0.00	-3.24	0.049	145**

$p < 0.05$; **$p<0.01$; minus sign indicates a decrease over control

Effect of temperature on the activity of *B. parabrevis* cellulase

Finding the effect of temperature on enzyme-substrate interaction

This experiment was done in two sets. For the first set organism was grown in a medium containing 0.5% cellulose as the major carbon source, and Whatman paper discs (5 discs of diameter 5 mm, amounting to 10.68±0.16 mg, in each experimental tube) were used as the substrate during enzyme assay. In the second set, organism was grown in a medium containing 2% CMC as the major carbon source, and 1% CMC was used as the substrate during enzyme assay. Temperature at which an incubation for 30 min was made allowing the enzyme to work on substrate ranged from 40-100°C. Cellulase activity obtained at different temp for both set of experiments is shown in Table 4 and Figure 1. In the first experimental set the cellulase activity was found to be maximum at 90°C. Enzyme activity at 40°C was statistically equivalent to that of 90°C. At all other temperatures the enzyme activity was found to be lesser (30-70% of that found at 90°C). In the second set of experiments, maximum enzyme activity was observed at 90°C, however the cellulase activity in the whole range of 40-100°C was almost similar. Though statistically significant but minor decrease in activity was obtained at 50°C and 60°C. This experiment was also performed with unpurified crude enzyme (i.e. the cell free supernatant used directly as an enzyme source), and maximum activity was observed at 90^{0}C (data not shown). The level of cellulase activity in second set (using CMC as the substrate for enzyme assay) of

experiments is almost double than that observed in the first set (using Whatman paper as the substrate for enzyme assay). This may possibly be due to different affinities of the *B. parabrevis* cellulase for two different substrates. Many factors contribute in determining how challenging cellulase activity assays can be. Few among these factors are the degree of homogeneity and purity of the cellulase sample; solubility of the substrate; the complicated relationship between physical heterogeneity of the cellulosic materials, and the complexity of cellulase enzyme systems (synergy and/or competition) (**Zhang *et al.*, 2015**).

Table 3 Cellulase activity over multiple subculturings of selected overproducing mutants of *B. parabrevis*

Colony designation	Cell density at harvest (OD_{660}) (Mean ± SD)	% Change compared to control	Cellulase activity (IU/ ml)	% Change compared to control
		1st generation		
Control	0.71±0.04	0.00	0.020	0.00
4A	0.72±0.05	1.81	0.050	150**
6A	0.74±0.06	4.60	0.039	95**
6B	0.73±0.02	2.93	0.047	135**
6C	0.69±0.00	-3.24	0.049	145**
		2nd generation		
Control	0.37±0.00	0.00	0.029	0.00
4A	0.42±0.00	12.5**	0.026	-10.34
6A	0.47±0.01	26.32**	0.045	55.17
6B	0.34±0.02	-9.30*	0.043	48.27*
6C	0.48±0.00	30.05**	0.036	27.58*
		3rd generation		
Control	0.66±0.00	0.00	0.030	0.00
4A	0.53±0.07	-19.88	0.042	40**
6A	0.61±0.00	-7.62**	0.042	40**
6B	0.52±0.02	-24.89**	0.039	30
6C	0.52±0.00	-24.74**	0.045	50
		4th generation		
Control	0.71±0.00	0.00	0.015	0.00
4A	0.76±0.02	7.11	0.017	13.33
6A	0.68±0.02	-4.18	0.023	53.33
6B	0.68±0.02	-4.04	0.022	46.66
6C	0.71±0.01	-0.13	0.020	33.33
		5th generation		
Control	0.52±0.00	0.00	0.021	0.00
4A	0.49±0.02	-5.71	0.022	4.76
6A	0.50±0.02	-3.23	0.022	4.76
6B	0.48±0.00	-7.61	0.026	23.80
6C	0.50±0.02	-3.61	0.026	23.80

*p<0.05; **p<0.01; minus sign indicates a decrease over control.

Cellulases capable of acting on their substrate at high temperatures are of interest for various industrial applications, as process is employing cellulases at higher temperature are less prone to contamination. Cellulolytic activity at high temperature is of special interest with respect to alcohol production from lignocellulosic wastes (**Van Maris *et al.*, 2006**). In the present study, we have found the cellulase activity to be optimum at a temperature as high as 90°C, this is particularly interesting considering that the cellulase producing organism used here is mesophilic with an optimum growth temperature of 35°C. This will also be interesting to investigate why a mesophilic bacterium need to produce a thermotolerant enzyme. Genes coding for such enzymes may also be useful while practicing genetic engineering for construction of other superior cellulase producing strains. Many microorganisms are known for their good cellulolytic potential, but their cellulases are functioning optimally at mesophilic temperatures. For example, Li *et al.* (2010) have shown optimum temperature CMCase activity from *Trichoderma viride* to be 50°C.

Table 4 Cellulase activity as a function of temperature.

Temp (°C)	SET I		SET II	
	IU /mL (Mean±SD)	% of maximum (considering IU at 90°C as cent percent)	IU/mL (Mean±SD)	% of maximum (considering IU at 90°C as cent percent)
40	0.050±0.000	92.59	0.104±0.000	95.41
50	0.037±0.000	68.51**	0.103±0.001	94.49*
60	0.038±0.001	70.37**	0.101±0.001	92.66*
70	0.018±0.001	33.33**	0.105±0.000	96.33
80	0.016±0.000	29.62**	0.102±0.003	93.57
90	0.054±0.001	100	0.109±0.000	100
100	0.023±0.002	42.59**	0.099±0.000	90.82

*p<0.05; **p<0.01;
Set I: Cellulose as growth substrate, and Whatman paper as substrate during enzyme assay.
Set II: CMC as growth substrate, as well as substrate during enzyme assay.

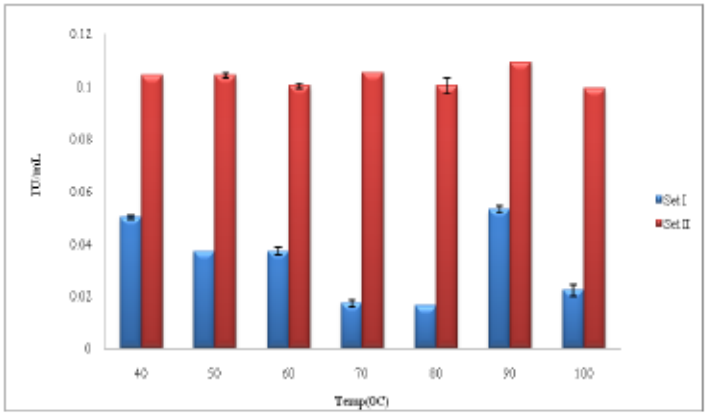

Figure 1 Cellulase activity as a function of temperature.
Set I Cellulose as growth substrate, and Whatman paper as substrate during enzyme assay.
Set II CMC as growth substrate, as well as substrate during enzyme assay

Investigating thermostability of the enzyme

After observing that the optimum temperature of the *B. parabrevis* cellulase is on the higher side, we proceeded for investigating whether this enzyme undergoes any structural modification after being subjected to heating. These experiments were performed in three different sets. Set I employed cellulose as the growth substrate, and Whatman paper as as the substrate during enzyme assay. Set II employed CMC as the growth substrate as well as substrate during enzyme assay. Set III employed CMC as the growth substrate, and Whatman paper as the substrate during enzyme assay. Here, we subjected the partially purified enzyme solution to heating at 50°C, 80°C, 100°C for 1 h, and 121°C (autoclaving) for 30 min. Results of the cellulase activity assay for the enzyme preparation(s) immediately after the heat treatment, and following cooling are presented in Table 5-7. Fluorescence spectrum of these cooled enzyme samples (for set I and II) were also generated.

Table 5 Cellulase activity of the heat denatured, and renatured enzyme preparation (set I).

Denaturation Temp (^{0}C)	Denaturation (in water bath for 1 h)		Renaturation (2 h at 35 ^{0}C)		
	IU /ml	% activity retained compared to Native	IU/ml	% diff. compared to denatured	% compared to Control
50	0.04±0.02	66	0.05±0.00	25	83.3
80	0.05±0.02	83.3	0.07±0.01	40	116.6
100	0.06±0.01	100	0.05±0.00	0	100.0
121 (30 min)	0.01±0.00	16*	0.04±0.00	300*	66

Cellulase activity of the control sample (not exposed to heat) was 0.06±0.00 IU/mL; *p<0.05

Table 6 Cellulase activity of the heat denatured, and renatured enzyme preparation (set II).

Denaturation Temp (^{0}C)	Denaturation (in water bath for 1 h)		Renaturation (2 h at 35^{0}C)		
	IU /ml	% activity retained compared to Native	IU/ml	% diff. compared to denatured	% compared to Control
50	0.099±0.001	90.00*	0.104±0.002	5.05	94.54
80	0.096±0.001	87.27*	0.100±0.003	4.16	90.90
100	0.098±0.001	89.09*	0.101±0.002	3.06	91.81
121 (30 min)	0.096±0.000	87.27*	0.100±0.002	4.16	90.90

Cellulase activity of the control sample (not exposed to heat) was 0.110±0.002IU/mL; *p<0.05.

Table 7 Cellulase activity of the heat denatured, and reantured enzyme preparation (set III).

Denaturation Temp (^{0}C)	Denaturation (in water bath for 1 h)		Renaturation (2 h at 35^{0}C)		
	IU /ml	% activity retained compared to control	IU/ml	% diff compared to denatured	% compared to control
50	0.223±0.000	83.52**	0.249±0.04	111.65*	93.25
80	0.229±0.007	85.76*	0.219±0.012	95.63	82.02*
100	0.182±0.012	68.16*	0.202±0.025	110.98	75.65
121(30 min)	0.237±0.015	88.76	0.229±0.001	96.62	85.76*

Cellulase activity of the control sample (not exposed to heat) was 0.267±0.005 IU/mL; *p<0.05; **p<0.01

During the experiments of set I, enzyme activity in the samples heated at 50-100°C was significantly not different than that in unheated control. However the autoclaved enzyme preparation suffered a loss of 83.34% activity compared to the control; this sample regained activity equivalent to the control after cooling. Thus except for autoclaving, the enzyme activity was not affected by heating. Though the activity of all the heat treated enzyme samples (after cooling) was at par to that of unheated control, structures of the reantured enzyme samples did not seem to be the same, as indicated by a shift in their fluorescence spectrum. Fluorescence spectrum of the native (unheated control) enzyme preparation exhibited an emission maxima at 341 nm, whereas this value was higher for the renatured enzyme samples (which were previously subjected to heat treatment) indicating a red shift (Fig. 2). For the experiments of set II, there was a minor (11-13%) decrease in the enzyme activity measured immediately after heat treatment; however following renaturation upon cooling the experimental enzyme preparations showed no significant difference with respect to their activity compared to the control. Fluorescence spectrum obtained in this set of experiments (spectrum not shown to avoid redundancy) was similar to that obtained for set I, exhibiting a red shift in the emission maxima of the heat treated enzyme preparations. For the third set of experiments, enzyme activity after heat treatment suffered maximum (32%) decrease at 100°C, however following cooling it regained the activity equivalent to that of control.

From the above described experiments regarding thermostability of the *B. parabrevis* cellulase, it is evident that this enzyme does not show any major significant reduction in its activity when measured immediately after heat treatment (i.e. retains considerable activity even after heat treatment), and upon

renaturation (after cooling) regains activity almost at par to that of native (unheated control). However the structure of this enzyme does undergo some change(s) upon heat treatment, as indicated by the fluorescence spectrum. But this altered structure also exhibits catalytic efficiency almost equivalent to that of native structure. It may be assume that the active site of this cellulase is not getting distorted even after structural alteration(s) owing to heat treatment, neither its accessibility to the substrate is reduced much. In our study the fluorescence spectrum of the heat treated enzyme preparation exhibited an increase in the emission maxima as compared to that of native, and the magnitude of this shift increased with increase in temp (Fig 2). This phenomenon of red shift can be considered as an indication of unfolding and denaturation of the enzyme structure, and revelation of more hydrophobic amino acid to the surface. The indole group of tryptophan is the dominant fluorophore in proteins, which absorbs near 280 nm, and emits near 340 nm. The emission of indole may shift to longer wavelengths (red shift) when the protein is unfolded (**Joseph *et al.*, 2006**). The indole group of tryptophan residues in proteins is a solvent-sensitive fluorophore, and the emission spectra of indole can reveal the tryptophan residues in proteins. The emission from an exposed surface residue is known to occur at longer wavelengths than that from a tryptophan residue in the protein's interior. This phenomenon apparent in Figure 2 is characterized by a shift in the spectrum of a tryptophan residue upon unfolding of a protein, and the subsequent exposure of the tryptophan residues to the aqueous phase. Before this unfolding, the residue is likely to be shielded from the solvent by the folded protein. Such unfolding of a cellulase may not always affect its substrate binding properties. Binding behavior of *Trichoderma reesei* celullases was shown not to be adversely affecting at temperature above 50^{0}C (**Andreaus *et al.*, 1999**). Tryptophan residue has been indicated as key amino acid in the structure of *T. reesei* cellulase (**Nakamura *et al.*, 2013**), as well as in cellulase of *Bacillus* species (**Ozaki *et al.*, 1991**). An increased frequency of exposed aromatic residues is believed to underline a stabilizing effect in enhancing thermal stability of proteins (**Chakravarty *et al.*, 2002**).

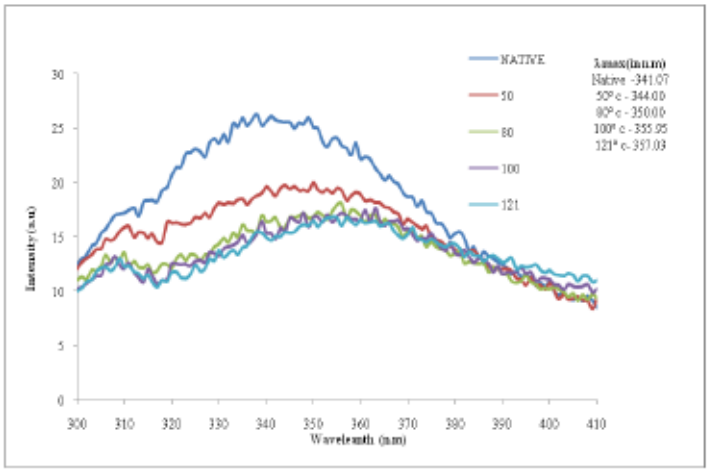

Figure 2 Structure of enzyme preparation after renaturation (Set I).

CONCLUSION

The study attempted at MW mutagenesis of *B. parabrevis* for enhanced production of thermostable cellulase. Though none of the four overproducing mutant lines obtained were found to be genetically stable, our study positively indicate the possible contribution of athermal MW effects towards their mutagenic potential. Investigation on the thermostability of the cellulase activity (performed with the wild type) revealed that the enzyme activity is not destroyed much even after heat treatment, despite certain structural changes (revealed from fluorescence spectrum) taking place owing to heat treatment. This enzyme was found to be catalytically active over a broad range of temperature. To the best of our awareness, this is the first description regarding thermostability of the *B. parabrevis* cellulase. More detailed investigation on this cellulase is warranted to understand the molecular basis underlining the thermostable nature of this protein. Cellulases capable of working at high temperature and retaining activity even after considerable heat exposure can find many interesting industrial applications (**Haki *et al.*, 2003**). It is noteworthy that this thermostable cellulase is not from a thermophilic organism, but from a mesophile. Mesophiles are easier to handle in lab as well as during large scale fermentative processes.

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