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

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

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Full Length Research Paper

First detection of *bla* TEM, SHV and CTX-M among Gram negative bacilli exhibiting extended spectrum β-lactamase phenotype isolated at University Hospital Center, Yalgado Ouedraogo, Ouagadougou, Burkina Faso

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Resistance to a wide variety of common antimicrobials is observed among clinical strains designed as extended spectrum β-lactmase (ESBL) producers. They produce enzymatic protein which inactivates efficiently oxyimino cephalosporin and constitutes a serious global health concern that has complicated treatment strategies. Many studies report high prevalence of ESBL producers among Gram negative bacilli. The aim of this work was to identify the presence of TEM, SHV and CTX-M families in these strains which were initially screened by phenotypic method. Gram negative bacilli resisting third or four generation cephalosporin were isolated during anti-biogram study. The presence of ESBL positivity was detected using the double disk synergy test. Minimal inhibitory concentrations (MICs) of ceftriazon for any strain were determined using E-test manufacturing protocol. Polymerase chain reaction (PCR) analysis for β-lactamase (*bla*) genes of TEM, SHV and CTX-M family was carried out using designed primers in 171 ESBL isolates producers. Among 259 Gram negative bacilli collected, 171 (66, 02%) exhibited ESBL producers' profile. Urine samples constitute major source of ESBL producers. The highest prevalence of ESBL was observed in *Escherichia coli* (75, 50%). Among ESBL isolates producers, gene prevalence of *bla*-CTX-M (65, 49%) was highest, followed by *bla*-TEM (25, 73%) and *bla*-SHV (18, 71%) in the present study. The frequency of ESBL producing strains among clinical isolates has been steadily increased. Continual drug resistance surveillance and molecular characteristics of ESBL isolates are necessary to guide the appropriate and judicious antibiotic use.

Key words: Extended spectrum β-lactamase (ESBL), double disk synergy test, $_{bla}$TEM, $_{bla}$SHV, $_{blaC}$TX-M, PCR.

INTRODUCTION

Loss of antibacterial proprieties is now established for many antibiotics substances, particularly those of β-lactam class with regard to therapy failure observed in clinical offices. For Gram negative bacilli, the most important

mechanism of resistance is based on the production of β-lactamases, enzymatic proteins, which hydrolyze β-lactam ring (Webb, 1984) described several decades later (Abraham and Chain, 1940). In Gram negative bacilli, β-lactamases are produced and stocked in periplasmic compartment (Frère, 1998). According to catalytic mechanism, two families of β-lactamases are distinguished (Frère, 1995; Matagne et al, 1999). The serine β-lactamases carrying an amino-acid residue-seryl in the catalytic site and metallo-β-lactamases are needed for their catalytic activity in the presence of metallic ion in active site. According to their molecular structure, β-lactamases are organized in class A, C, D and B enzymes (Ambler, 1975, 1978, 1980). Serine β-lactamases consist of class A, C and D enzymes while metallo-β-lactamases consist of class B enzymes. Our study concerns class A enzymes which consist of the following groups: Temoneira (TEM), sulfidrhyl-variable (SHV), cefotaximase (CTX-M), pseudomonas extended resistance (PER). Those chromosomal or plasmid-mediated enzymes are either penicillinases (TEM-1/2 and SHV-1) hydrolyzing penicillins, first and second generation cephalosporins or extended-spectrum-β-lactamases (ESBL). Extended-spectrum-β-lactamases (TEM-3, SHV-2 and CTX-M), a form of β-lactamases, hydrolyzing third generation of cephalosporins (like oxy-iminocephalosporins); detected since 1983 (Knothe et al., 1983) are now worldwide reported. ESBL, detected at first in *Klebsiella pneumoniae* and *Escherichia coli* (Medeiros, 1984; Bradford, 2001), is now fully spread in a number of enterobacterial spices and other kind of bacterial isolates, often responsible for nosocomial epidemic outbreak. Therapy failure due to these fastidious strains makes clinicians to prescribe more and more carbapenem antibiotics for fighting resistant strains resisting all class of antibiotic (Queenan and Bush, 2007; Poirel et al., 2007). Therefore, a research on how to control ESBL epidemiology is a noble undertaking. Molecular studies leading in many countries have allowed getting more information on genetic matrix and molecular types. In Turkey, a survey of *Klebsiella* spp. from intensive care units from eight hospitals showed that 58% of 193 isolates harbored ESBLs (Gunseren et al., 1999). In Chicago, infections with TEM-type ESBLs particularly TEM-10, TEM-12, and TEM-26 were reported since 1996 (Schiappa et al., 1996). Boyd et al. (2004) in their study, described CTX-M-type ESBLs in United States and Canada. CTX-M-12 was first detected in Kenya by Kartali et al. (2002) (Kartali et al., 2002). Characterization of ESBLs from South Africa revealed the types of TEM and SHV (especially SHV-2 and SHV-5) (Hanson et al., 2001). In Burkina Faso, *bla* SHV and *bla* TEM were first reported in 2004 in community medical center (Zeba et al., 2004). Microbial drug resistances based on ESBL were reported (Karou et al., 2009), but there is no study on determining the different types of gene encoding. Therefore, this study aimed to contribute to clear bacterial resistance epidemiology by establishing genetic profile of Gram negative bacilli that resist oxy-iminocephalosporins and exhibit extended-spectrum β-lactamase at University Hospital Center Yalgado, Ouedraogo.

MATERIALS AND METHODS

Clinical specimens and strains

Bacterial strains collected during a prospective study from July 2009 to March 2012 were Gram negative bacilli that resist third generation of cephalosporin. They were isolated and diagnosis analysis of biological specimens was done. Different clinical specimens such as blood, urine, pus, ascitic fluid, peritoneal fluid, stool and rachis fluid samples were collected from different hospitalized patients. Samples were taken from infected patients who presented infectious evident symptoms like fever and purulent urine. Isolates were identified using conventional method (Kelly et al., 1985). Identification of isolates was achieved using API 20E test trips (BioMerieux S.A., Marcy l'Etoile, France).

Antibiotic susceptibility testing and ESBL detection

Antibiotic susceptibility was tested by disk diffusion method (Bauer, 1966), with antibiotic disks used to test Gram negative bacilli particularly monobactam: Aztreonam (30 µg), third generation cephalosporin like cefotaxim (30 µg), ceftriaxon (30 µg), ceftazidim (30 µg), and fourth generation cephalosporins: cefepim (30 µg). Antibiotics were tested on Petri plates containing Muller Hinton agar. Measurements of inhibition area determine the clinical categories (CA-SFM, 2010; 2011; 2012). Isolates that were resistant to at least one of the antibiotics in clinical test, using NCCLS methods (NCCLS, 2000) were collected, purified and conserved at -80°C for furthers analysis. In order to screen ESBL phenotypical profile, isolates were submitted for synergy test (Jarlier et al., 1988) between third generation of cephalosporins disks (cefotaxime or ceftazidime) and amoxicillin plus clavulanic acid.

In addition, MICs of ceftriaxone, antibiotic frequently used in clinical routine in our sanitary centers was performed as recommended by guide E-test AB BIODISK.

Molecular characterization of ESBL producing-isolates

All isolates which were positive for synergy test were screened in order to detect β-lactamase encoding genes for *bla* TEM, SHV and CTX-M families by previously described PCR protocols (Olivier et al., 2002; Pagani et al., 2003). DNA template was prepared from purified bacteria grown overnight at 37°C on Muller Hinton agar plates. Crude DNA extracts were obtained by suspending a colony in 100 µl of purified water and boiling at 95°C for 10 mn (Munday et al.; 2004). Mixed PCR for one reaction has the following components: 5 µl of Green buffer 5X; 1 µl of each Primer 10µM; 0.65µl of dNTPs 10 mM; 0.12 µl of Gotaq 0.5 U/µl and 15.25 µl of purified water. Reaction volume is completed to 25 µl with 2 µl of

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Table 1. Primers used for amplification of *BlaTEM*, *SHV* and *CTXM* genes.

Gene	Primer	Sequence (5'-3')	Amplicons size (bp)	References
blaTEM	TEM-F	ATAAAATTCTTGAAGACGAAA	1080	Olivier et al. (2002)
	TEM-R	GACAGTTACCAATGCTTAATCA		
blaSHV	SHV-F	ATGCGTTATATTCGCCTGTG	1030	Olivier et al. (2002)
	SHV-R	TTAGCGTTGCCAGTGCTC		
BlaCTX-M	CTX-F	GTTACAATGTGTGAGAAGCAG	1041	Pagani et al. (2003)
	CTX-R	CCGTTTCCGCTATTACAAAC		

Figure 1. Detection of ESBL *Escherichia coli* producer on plate. a) Pure colonies of *Escherichia coli.* b) Antibiogram of *Escherichia coli.* c) Synergy test showing ESBL *E. coli* producer on Petri plate.

DNA template. Primers supplied by Promega according to each β-lactamase gene type are in Table 1. PCR was carried out under the following conditions on SensoQest Labcycler, GmbH, Germany: initial denaturation step at 96°C for 5 min, followed by 35 cycles consisting of denaturation at 96°C for 1 min, annealing at 58°C, 60°C and 50°C for TEM, SHV and CTX-M at 1 min, primer extension at 72°C for 1 min and final extension for 10 min.

Migration of PCR products watched by Green buffer (Eugentec) during 30 min at 80 V was performed on agar gel of 1%, prepared with BET at 0. 25% as final concentration.

RESULTS

Bacterial strains and antibiotic susceptibility testing

During this study, 259 Gram negatives bacilli which resist at least one- third or fourth generation of cephalosporins or monobactam were collected from different samples (Figure 2). For all the strains collected, diameters of inhibiting area around oxy-iminocephalosporins disc were less than 16 mm. Resisting bacterial species isolated were about quantitative importance: 132 *Escherichia coli, 43 Klebsiella pneumoniae, 34 Pseudomonas aeruginosa, 24 Enterobacter* sp.,*11* Citrobacter spp *7 Accinetobacter baumannii,* 6 *Proteus mirabilis*, and 1 *Salmonella typhi*. Extended-spectrum β-lactamase phenotype screening showed that 171 strains (66, 02%) were positive for synergy test, suggesting that they were extended-spectrum- β-lactamases-producers (Figure 1). The highest proportion (62.57%) of their strains was isolated from urine samples.

The MICs of ceftriaxone determined by E-test were less than 50, 100 and 256 µg/ml for 81, 57, 55.26 and 39.74% isolates (Figure 2). MICs of ceftriaxone for 73.33% of *E. coli* (22/30) and 80% of *K. pneumoniae* were less than 64 µg/ml. Among the positive isolates for synergy test, bacterial species were spread as follows: 99 *E. coli* (that is, 75% of *E. coli*)*, 28 K. pneumonia* (that is, 65.11% of *K. pneumoniae*)*,* 19 *P. aeruginosa (*55.88%) *15 enterobacter* sp. *(*62,50%), 4 *citrobacter sp(*36,36%), 2 *acinetobacter baumannii*, 3 *Proteus mirabilis*, 1 *Salmonella typhi*. Except *P. aeruginosa* and *Accinetobacter baumannii, t*he rest isolates belong to enterobacterial family. The study focused on bacterial resistance and no process determined community or hospital origin of isolates.

Polymerase chain reaction: Amplification of *bla* genes

In the electrophoresis of PCR products realized with DNA ladder, positive and negative control showed bands as previously around 875 bp for *bla* SHV and 1000 bp for *bla* TEM CTX-M (Figure 3). Molecular characterization of our

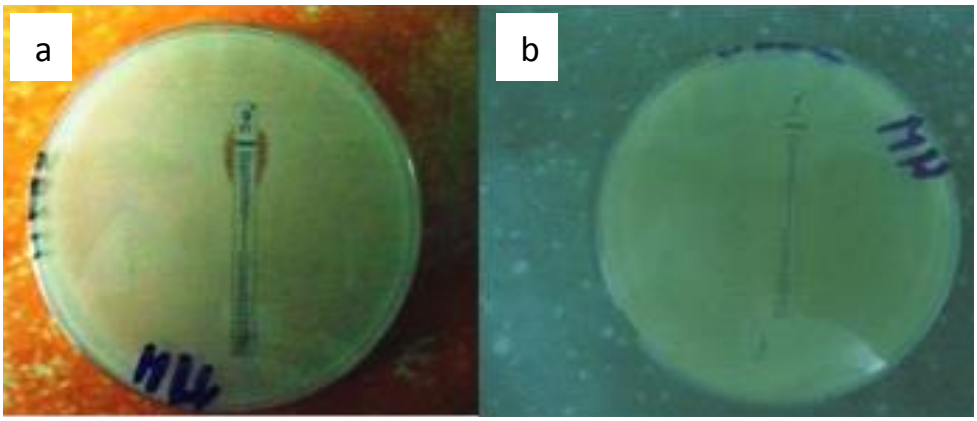

Figure 2. a. Sensitive pattern of *Escherichia coli* Ceftriazon, CMI = 96 µg/ml. b) Sensitive pattern of *Klebsiella pneumonia* Ceftriazon, CMI = 256 µg/ml.

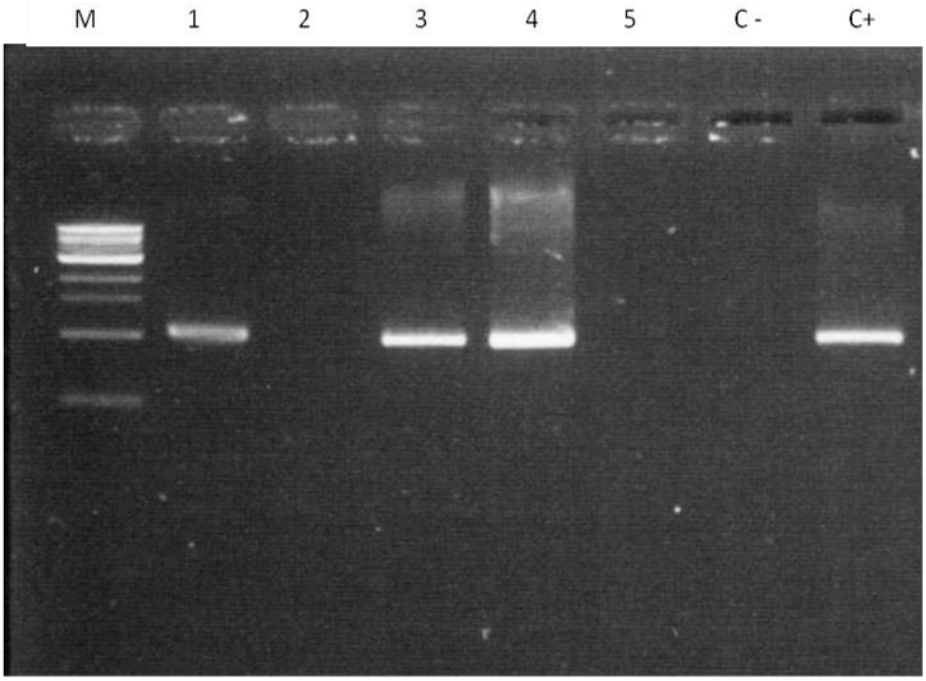

Figure 3. Agarose gel of CTX-M PCR products. M, Marker of molecular weight (GeneRuler 100 bp DNA ladder). Number 1-5 correspond to samples: 1 = 45H, 2 = 219P, 3 = 310P, 4 = 315 Uro, 5 = 225H. C+ = Positive control and C- = negative control.

isolates (exhibiting extended –spectrum β-lactamase phenotype) showed that 112 strains (65.49%) were positive for CTX-M screening; 44 stains (25.73%) for TEM screening and 32 strains (18.71%) for SHV. In all the bacterial species, some associations were found between the three types of *bla* genes. Eleven (11) isolates carried *bla TEM, SHV, CTX-M* at the same time. Finally, 134 isolates (78, 36%) were positive for β-lactamase gene screening and 37 strains did not carry any of their β-lactamase genes. Repartition of β-latamase gene type among bacterial species was established.

Among 99 *E. coli* investigated, 11.11% carried single *bla* TEM; 7.07% carried *bla* SHV and 48.48% carried single *bla* CTX-M. 5. 05% harbored *bla* TEM linked to *bla* SHV; 5.05% carried *bla* TEM linked to *bla* CTX-M; 5.05% carried *bla* SHV with *bla* CTX-M and 6.06% carried both *bla* TEM, *bla* SHV and *bla* CTX-M. 70.70% of *E. coli* carried at least *bla* CTX-M or CTX associated with other *bla* genes. Among 28 *K. pneumonia* screened for *bla* genes, 14.28% carried single *bla* TEM; 10. 71% carried *bla* SHV; 25% carried single *bla* CTX-M and 10.71% carried *bla* TEM associated with *bla* SHV. 3.57% strains

Table 2. Spread of *bla*-genes among bacterial spices.

Bacterial species	Number	TEM	SHV	CTX-M	TEM -SHV- CTXM
Escherichia coli	99	25	20	70	6
Klebsiella pneumoniae	28	9	4	12	1
Enterobacter sp.	15	3	5	10	2
Pseudomonas aeruginosa	19	5	2	12	2
Citrobacter sp.	4	1	1	3	0
Acinetobacter baumannii	2	1	0	2	0
Proteus mirabilis	3	0	0	2	0
Salmonella typhi	1	0	0	1	0
Total	171	44	32	112	11

carried *bla* TEM, *bla* SHV and bla CTX-M. 42.85% carried at least *bla* CTX-M.

From 15 *Enterobacter* sp. screened, 13.33% of strains carried *bla* SHV, 20% carried *bla* TEM associated with other genes, 20% carried *bla* SHV in association with *bla* CTX-M. 66.66% carried either *bla* CTX-M or CTX-M linked to other genes. Among 19 strains of *P. aeruginosa*, 10.52% harbored *bla* TEM, 31.57% carried *bla* CTX-M. 21.05% of strains carried *bla* TEM associated with *bla* CTX-M, while 5.26% carried *bla* CTX-M associated with *bla* SHV and 10.52% of strains harbored *bla* TEM, *bla* CTX-M and *bla* SHV. At least, *bla* CTX-M was found in 52.63% of *P. aeruginosa* screened. The distribution of *bla* genes among the bacterial species is shown in Table 2.

Our study has noted that patients of all age were affected by *bla* gene and ESBL (Table 3). From the results, 11.36, 9.37% and 5.35% of *bla* TEM, SHV and CTX-M, respectively were found on patients of less than 1 year old. For children of less than five years old, 22, 28.12 and 14.28% of *bla* TEM, SHV and CTX-M were found. According to sexual repartition, 40,90%, 50% and 44,64% of *bla* TEM, SHV and CTX-M were found in female group, while 65.90, 50 and 55.35% of *bla* TEM, SHV and CTX-M were found in male group.

DISCUSSION

The most important part of our strains was resistant to cefotaxim, ceftriaxon and aztreonam. The rate of resistance to those antibiotics was globally greater than 50% of isolates. Bacteriological and susceptibility study at Tunis University Hospital reported rates of resistance to β-lactam by enterobacterial at 57.90% (Larabi et al., 2003). PCR results have shown that most proportion of strains carried *bla* CTX-M which are ESBLs (Bonnet, 2004). There is high possibility that *bla* TEM and *bla* SHV are found in ESBL because producing stains were resistant to oxi-iminocephalosporin (Livermore, 2008). ESBLs have become a widespread serious problem. These enzymes are becoming increasingly expressed by many strains of pathogenic bacteria with a potential for dissemination. The presence of ESBL compromises the activity of wide-spectrum antibiotics creating major therapeutic difficulties with a significant impact on the outcome of patients. The continuous emergence of ESBLs presents diagnostic challenges to the clinical microbiology laboratories.

In this study, urine represent samples indexed as extended-spectrum β-lactamase isolates producing source. Similar observations are reported in others studies (Shanthi and Sekar, 2010; Iraj and Nilufar, 2011; Saba et al., 2012). Invasive treatment using catheter at hospital centers often explains high prevalence of urinary infections (Cantón et al., 2008). In addition, in community, people practice auto medication with antibiotics that can select ESBLs isolates which may be carried at hospital notably in emergencies units and disseminated in other clinical offices (Mbutiwi et al., 2013). In this study, it was found that *E.coli* is frequently identified as ESBL producing isolate (Shanthi and Sekar, 2010; Abhilash et al., 2010; Umadevi et al., 2011). It is bacterial species, most adapted to and represented in digestive stratus and consequently receives plasmid-mediated gene from other bacteria.

Phenotypic tests for ESBL detection only confirm if an ESBL is produced but cannot detect the ESBL subtype. Some ESBLs may fail to reach a level to be detected by disk diffusion tests but result in treatment failure in the infected patient. Nuesch-Inderbinen and Hachler (1996) reported that molecular methods appear sensitive, are expensive, time consuming and require specialized equipment and expertise. However, definitive identification is possible only by molecular detection methods.

There are so many types of β-lactamase like TEM, SHV, CTX, OXA, AmpC, etc, but majority of the ESBLs are derivatives of TEM or SHV or CTX-M enzymes and these enzymes are most often found in *E. coli* and *K. pneumonia* (Paterson and Bonomo, 2005). Keeping in view this fact, the current study investigated Gram negative bacilli among which *E. coli* and *K. pneumoniae* were most represented. The study aimed to look for the presence of *TEM*, *SHV* or *CTX-M* gene.

From the results of the study, the three families of β-lactamase genes screened were found with high prevalence of CTX-M, followed by TEM and SHV. PCR

Table 3. Spread (number) of *bla* genes by sex/age on infected patients.

Age (years)	<1		1-		5-14		15 -25		≥26	
Sex / *Bla*	M	F	M	F	M	F	M	F	M	F
TEM	03	02	03	02	02	03	04	02	17	09
SHV	01	02	02	04	03	00	02	03	08	07
CTX	03	03	04	06	03	02	05	08	47	31

screening of 99 *E. coli* has shown that 25.25% harbored *TEM* gene, 20.20% harbored *SHV* gene and 70.70% harbored *CTX-M* gene. Of 28 *K. pneumonia*, 14.28% harbored *TEM* gene, 32.14% harbored *SHV* gene and 42.85% harbored *CTX-M* gene. 20% of *Enterobacter* sp. harbored *TEM* gene, 33.33% harbored *SHV* gene and 66.66% harbored *CTX-M* gene. 26.31% *Pseudomonas aeruginosa* harbored TEM gene, 10,52% harbored SHV gene and 63,15% harbored CTX-M gene. Livermore et al., stated in separate studies that the CTX-M gene is the most prevalent ESBL-encoding gene worldwide and is replacing TEM and SHV types as the predominant ESBL in many European and Asian countries (Livermore et al., 2007; Bali et al., 2010). The high prevalence of CTX-M gene in our study is in concordance with the study of other authors (Vaida et al., 2010) who reported CTX-M-encoding genes in the majority of *E. coli* (96%) and *K. pneumoniae* (71%) isolates showing ESBL phenotype. In our study, major part of the isolates carried more than one type of gene. Among *E.coli* , *bla* TEM, *bla* SHV and *bla* CTX-M matched with each other in the same proportion(5,05%). Among Klebsiella pneumonia, *bla* TEM associated with *bla* SHV in 10, 71% of strains. 6, 43% isolates harbored all the three β lactamase genes. CTX-M is frequently associated with other β lactamase genes. According to Goyal et al., majority of strains (57.3%) harbored two or more ESBL genes. Bali et al. have observed in their study that 19.2% ESBL isolates carried more than one type of β lactamase genes (Goyal et al., 2009; Bali et al., 2010). The three types of *bla* gene were found within group of children of less than 5 years old. This fact is relevant for using antibiotics for viral infections, that are resistant (Bergus et al., 1996). The limitation of our study is the bacterial ecology which was not identified by any process. We were not able to know for some patients, if resistant bacteria were acquired in the community or hospital.

Conclusion

Our study has shown high prevalence of β-lactamases-isolates producers among clinical strains detected through analysis of biological samples collected in different units of University Hospital Center, Yalgado OUEDRAOGO. CTX-M type (ESBL) was mostly represented, followed by TEM and SHV β-lactamases. Escherichia coli, like in other several studies, was the strain that expressed ESBL frequently and can associate with more than one type. *P. aeruginosa* nosocomial, fastidious strain, known for producing PER currently harbored more and more CTX-M, TEM and SHV. This fact increases risk of ESBL dissemination between plasmid receptive strains or/and between clinical units. The present study has established that β-lactamin antibiotic except cephamycin and carbapenem (imipenem) was sensitive to ESBL. Diameters of inhibition area of oxy-iminocphalosporin (cefotaxim, ceftriazon, ceftazidim) for our isolates were seriously reduced, currently less than 20 mm. ESBL strains are usually multi-drug resistant. So, the practice of routine ESBL testing along with conven-tional antibiogram would be useful for all cases; it will help in theproper treatment of patients and also prevent further development of bacterial rug resistance. Molecular detection and identification of β- lactamases would be essential for a reliable epidemiological investi-gation of antimicrobial resistance. It is necessary to control hygiene and antibiotics consummation in hospital centers.

Conflict of interests

The authors did not declare any conflict of interest.

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REFERENCES

Abhilash KP, Veeraraghavan B, Abraham OC (2010). Epidemiology and Outcome of Bacteremia Caused by Extended Spectrum Beta-Lactamase (ESBL)-producing *Escherichia Coli* and *Klebsiella* Spp. in a Tertiary Care Teaching Hospital in South India. J. Assoc. Physicians India 58:13-7.

Abraham EP, Chain E (1940). An enzyme from bacteria able to Destroy Penicillin. Nature London 146:837.

Ambler RP (1975). The aminoacid sequence of staphylococcus aureus pnicillinase. Biochem.J.151:197-218
Ambler RP (1980). The structure of β-lactamases.Philos. Trans. R. Soc. London Ser. B. 28:321-331
Ambler RPK Scott (1978). Partial aminoacid sequence of penicillinase coded by Escherichia coli plasmid R6K. Proc. Natl. Acad. Sci. USA. 75:3732-3736
Bali EB, Ac KL, Sultan N (2010). Phenotypic and molecular characterization of SHV, TEM, CTX-M and extended-spectrum-lactamase produced by *Escherichia coli*, Acinobacter baumannii and *Klebsiella isolates* in a Turkish hospital. Afr. J. Microbiol. Res. 4(8):650-54
Bauer AW, Kirby WMM, Sherris JC, Tuck M (1966). Antibiotics susceptibility testing by standardized single disc method. Am. J. Clin. Pathol. 45:493-65
Bergus GR, Levy BT, Levy SM, Slager SL, Kiritsy MC (1996). Antibiotic use during the first 200 days of life. Arch. Fam. Med. 5:523-6. 14.
Bonnet R (2004). Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. Antimicrob. Agents Chemother. 48:1-14.
Boyd DA, Tyler S, Christianson S, McGeer A, Muller MP, Willey BM, Bryce E, Gardam M, Nordmann P, Mulvey MR (2004). Complete nucleotide sequence of a 92-kilobase plasmid harboring the CTX-M-15 extended-spectrum beta-lactamase involved in an outbreak in long-term-care facilities in Toronto, Canada. Antimicrob. Agents Chemother. 48:3758-3764
Bradford PA (2001). Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology and detection of this important resistance threat. Clin. Microbiol. Rev. 14:933-51
Cantón R, Novais A, Valverde A, Machado E, Peixe L, Baquero F, Coque T(2008)Prevalence and spread of extended-spectrum β-lactamase-producing Enterobacteriaceae in Europe. Clin. Microbiol. Infect. 14:144-153
Frère JM (1998). Résistance bactérienne aux antibiotiques : le role prépondérant des β-lactamase. Bulletin de la classe des Sciences Académie Royale de Belgique 6ème série tome IX 139-146
Frère JM (1995). β-lactamases and bacterial resistance to antibiotics. Mol. Microbiol. 16:385-395.
Goyal A, Prasad KN, Prasad A, Gupta S, Ghoshal U, Ayyagari A (2009). Extended spectrum beta-lactamases in *Escherichia coli* & Klebsiella pneumoniae & associated risk factors. Indian J. Med. Res. 129(6):695–7001
Gunseren F, Mamikoglu L, Ozturk S, Yucesoy M, Biberoglu K, Yulug N, Doganay M, Sumerkan B, Kocagoz S, Unal S, Cetin S, Calangu S, Koksal I, Leblebicioglu H, Gunaydin M (1999). A surveillance study of antimicrobial resistance of gram-negative bacteria isolated from Intensive care units in eight hospitals in Turkey. J. Antimicrob. Chemother. 43:373-378
Hanson ND, Moland ES, Pitout JD (2001). Enzymatic characterization of TEM-63, a TEM-type extended spectrum beta-lactamase expressed in three different genera of *Enterobacteriaceae* from South Africa. Diagn. Microbiol. Infect. Dis. 40:199-201.
Iraj A, Nilufar YN (2010). Antibiogram of Extended Spectrum Beta-lactamase(ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae* isolated from Hospital Samples. Bangladesh J. Med. Microbiol. 4(1):32-36.
Jarlier V, Nicoals M-H, Fournier G, Philipon A (1988). Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in enterobacteriaceae: hospital prevalence and susceptibility patterns. Rev. Infect. Dis. 10:867-878
Karou SD, Denise PI, Wendyame MC, Nadembega WM, Yaovi A, Djeneba O, Salvatore P, Virginio P, Alfred ST, Comlan DS Jacques S, (2009). Antibiotic Resistance in Urinary Tract Bacteria in Ouagadougou. Pak. J. Biol. Sci. 12:712-716.
Kartali G, Tzelepi E, Pournaras S, Kontopoulou C, Kontos F, Sofianou D, Maniatis AN, Tsakris A (2002). Outbreak of infections caused by *Enterobacter cloacae* producing the integron-associated beta-lactamase IBC-1 in a neonatal intensive care unit of a Greek hospital. Antimicrob. Agents Chemother. 46:1577-1580.
Kelly MT, Brenner DJ, Famer JJ III (1985). Anterobacteriaceae. In: Lemette EH Ballows A, Hausler WJ Jr, Shadomy HJ, editors. Manual of clinical microbiology. Washington, DC: American society for Microbiology. pp. 263-277.
Knothe GP, Shah P, Kremery V, Antai M, Mitsuhashi S (1983). Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of Klebsiella pneumoniae and Serratia marcescens. Infection 11:315-317
Larabi K, Masmoudia A, Fendri C (2003). Bacteriological and susceptibility study of 1930 strains isolated from UTIs in Tunis University Hospital. Med. Mal. Infect.33:348-352
Livermore DM (2008). Defining an extended-spectrum β-lactamase. Clin. Microbiol. Infect.14:3-10
Livermore DM, Canton R, Gniadkowski M, Nordmann P, Rossolini GM, Arlet G, et al.(2007). CTX-M: changing the face of ESBLs in Europe. J. Antimicrob. Chemother. 59:165-74.
Matagne A, Dubus A, Galleni M, Frère J-M (1999).The β-lactamase cycle: a tale of selective pressure and bacterial ingenuity Nat. Prod. Rep. 16(1):1-19.
Mbutiwi FIN, François LB, Michèle DW, Philippe M, Myriam M, Nazaire NM, Flory MT, Dramane K (2013). L'automédication chez des patients reçus aux urgences médicales des Cliniques Universitaires de Kinshasa. CAIRN.INFO. Santé Publique. 2(25):118
Medeiros AA (1984). Betalactamase. Br. Bull. 40:18-27.
Munday CJ, Xiong J, Shen CLD, Hawkey PM (2004). Dissemination of CTX-M type -lactamases in Enterobacteriaceae isolates in the People's Republic of China. Int. J. Antimicrob. Agents 23:175-180.
NCCLS (2000). Methods performance standards for antimicrobial disk susceptibility test approved standards. p.M2-A7
Nuesch-Inderbinen MT, Hachler FHKH (1996). Detection of genes 15 coding for extended-spectrum SHV beta-lactamases in clinical isolates by a molecular genetic method, and comparison with the Etest. Eur. J. Clin. Microbiol. Infect. Dis. 15:398-402.
Oliver A, Weigel LM, Rasheed JK, McGowan JE, Raney P, Tenover FC (2002). Mechanisms of decreased susceptibility to cefpodoxime in *Escherichia coli*. Antimicrob. Agents Chemother. 46(12):3829–3836.
Pagani L, Dell'Amico E, Migliavacca R (2003). Multiple CTX-M-type extended-spectrum *β*- lactamases in nosocomial isolates of *Enterobacteriaceae* from a hospital in Northern Italy. J. Clin. Microbiol. 41(9):4264-4269.
Paterson D, Bonomo R (2005). Extended-spectrum β-lactamases: a clinical update. Clin. Microbiol. Rev. 18:657-86
Poirel L, Naas T, Nordmann P (2010). Diversity, Epidemiology, and Genetics of Class D-Lactamases. Antimicrob. Agents Chemother. 54(1):24-38.
Queenan AM, Bush K (2007). Carbapenemases: the versatile beta-lactamases. Clin. Microbiol. Rev. 20:440–458.
Saba R, Muhammad F, Shahida H(2012). Prevalence and comparison of Beta-lactamase producing *Escherichia coli* and *Klebsiella spp.* from clinical And environmental sources in Lahore, Pakistan. Afr. J. Microbiol. Res. 6(2):465–670.
Schiappa DA, Hayden MK, Matushek MG, Hashemi FN, Sullivan J, Smith KY, Miyashiro D, Quinn JP, Weinstein RA, Trenholme GM (1996). Ceftazidime-resistant *Klebsiella pneumoniae* and *Escherichia coli* bloodstream infection: a case-control and molecular epidemiologic investigation. J. Infect. Dis. 174:529-536.
Shanthi M, Sekar U (2010). Extended Spectrum Beta Lactamase Producing*Escherichia Coli* and *Klebsiella Pneumoniae*: Risk Factors for Infection and Impact of Resistance on Outcomes. J. Assoc. Physicians India 58 Suppl:41-44.
Umadevi S, Kandhakumari G, Joseph NM, Kumar S, Easow JM, Stephen S, Singh UK (2011). Prevalence and antimicrobial Susceptibility pattern of ESBL producing Gram Negative Bacilli. J. Clin. Diagn. Res. 5(2):236-239.
Vaida S, Marius L, Aurelija B, Justas P, Rita P, Agne G (2010). Molecular characterization of extended-spectrum b-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* isolates from hospitals in Lithuania. J. Med. Microbiol. 59(10):1263-1265.
Webb EC (Ed) (1984). Enzyme Nomenclature. Academic press Inc. (London Ltd London). pp. 366-374.
Zeba B, Simpore J, Nacoulma OG, Frere JM (2004). Prevalence of blaSHV genes in clinical isolates of Klebsiella pneumoniae at Saint Camille medical center in Ouagadougou. Isolation of blaSHV11 like gene. African journal of Biotechnology vol.3(9),pp.477-480.

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Full Length Research Paper

Detection of *Pseudomonas fluorescens* from broth, water and infected tissues by loop-mediated isothermal amplification (LAMP) method

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Loop mediated isothermal amplification is rapid, highly sensitive and specifically developed method for detection of bacterial infections. AprX gene for alkaline metalloprotease of *Pseudomonas fluorescens* was used to design four primers and loop mediated isothermal amplification (LAMP) conditions were standardized for amplification of DNA. LAMP primers successfully amplified *P. fluorescens* from DNA and bacterial cells taken directly from broth, water and infected tissues with high specificity and sensitivity (10 pg) under isothermal condition at 61°C.

Key words: *Pseudomonas fluorescens*, loop mediated isothermal amplification (LAMP), rapid, simple, specificity, sensitivity.

INTRODUCTION

Pseudomonas fluorescens is a member of the fluorescent pseudomonad group and (unlike *Pseudomonas aeruginosa*) has generally been regarded to be of low virulence and an infrequent cause of human infection (Hsueh et al., 1998). But later on, six strains grown in transfusion blood having characteristic features of *P. fluorescens* had caused severe to fatal reactions in the recipients (Pittman, 1953). Sutter (1968) also isolated *P. fluorescens* repeatedly from the blood of a patient with an abdominal abscess following bowel resection. *P. fluorescens* has been reported to cause occasional cases of transfusion-associated septicemia in blood recipients, including fatal reactions (Scott et al., 1988), and catheter-related bacteremia in patients with cancer (Hsueh et al., 1998). *P. fluorescens* is an aerobic, rod shaped, Gram-negative bacteria that grows best at temperature range between 25 and 30°C.

In 2001, *P. fluorescens* was also isolated from carp in the Abbassa fish farm, with a prevalence rate of 23% (Aly, 2001) while during 2002, *P. fluorescens* was isolated from Nile tilapia cultured in duck-fish farms at Ismailia and Sharkia Provinces with prevalence of 8% (Aly et al., 2002). In 2004, seven out of the 17 commercial fish farms in Kafr ElSheikh Governorate suffered from high mortalities, ranging from 17.6 to 22.9%. Bacteriological examinations revealed 36.9% fish were infected with *P. fluorescens*, 29.1% with *P. aureginosa*, 18.5% with *Pseudomonas anguilliseptica* and 15.5% with *Pseudomonas pseudoalkaligene* (Masbouba, 2004).

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Eissa et al. (2010) isolated different strains of *Pseudomonas* species namely *Pseudomonas putida*, *P. aeruginosa*, *P. fluorescens* and *P. anguilliseptica* from *Oreochromis niloticus* in Qaroun and Wadi-El-Rayan Lakes, Egypt. They reported that infected fishes showed irregular hemorrhages on body surface, especially at the ventral part of abdomen, eyes cloudiness, scales detachment and congested gills were observed. Internally, there were sanguineous fluids in the abdominal cavity of some fish. Also, these results are in agreement with those of EL-Hady and Samy (2011) who isolated *P. putida*, *P. aeruginosa*, *P. fluorescens* and *P. anguilliseptica* from cultured *O. niloticus* and *Cyprinus carpio* from different fish farms at different localities in Egypt. This data clearly indicates that infection is responsible for huge losses to aquaculture.

Pseudomonas spp. causes septicaemia in aquatic animals (Roberts, 1978) and a number of aquatic animals including fish, frogs and soft-shelled turtles are reported to be susceptible to *Pseudomonas* spp. with moderate to high losses (Somsiri and Soontornvit, 2002). *Pseudomonas* spp. bacteria are considered as opportunistic indoor pathogens as their infection initiates an inflammatory response (Hirvonen et al., 2005; Huttunen et al., 2003). Bacteria invades the host tissue and cause infection and bacteremia in immunocompromised hosts (HIV/AIDS, cystic fibrosis, bronchiectasis, severe chronic obstructive pulmonary disease, burns, malignancy or diabetes mellitus) (Feldman et al., 1998; Liu and Mercer, 1963). Identification of *P. fluorescens* is very tedious as it is not a frequent cause of human infections; prefers versatile environments (like water, soil, foods, etc.) and grow poorly at the standard hospital microbiology incubation temperature of approximately 36°C (Weyant et al., 1996; CDC, 2005).

Detection of *P. fluorescens* by standard plate count method, polymerase chain reaction (PCR) and RT-PCR is time consuming and also such methods require the use of special equipments. On the other hand, loop mediated isothermal amplification (LAMP) (Notomi et al., 2000; Nagamine et al., 2002) is simple, easy, rapid and cost-effective method which have been widely used for the diagnosis of bacteria (Gahlawat et al., 2009; Chen at al., 2011; Han et al., 2011; Saharan et al., 2014a), viruses (Wang et al., 2011; Zhao et al., 2011; Saharan et al., 2014b) and parasites (Ikadai et al., 2004; Iseki et al., 2007; Lu et al., 2011). Seeking the benefits of LAMP method, the present study is planned to determine application of LAMP assay for rapid detection of *P. fluorescens* from DNA and bacterial cells taken directly from broth, water and infected tissues.

MATERIALS AND METHODS

Bacterial strains

Aeromonas hydrophila (MTCC 646), *Lactobacillus acidophilus* (MTCC 447), *P. flourescens* (MTCC 7200), *Staphylococcus aureus* (MTCC 87) and *Bacillus cereus* (MTCC 6728) were purchased from Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Microbial Technology Chandigarh (India) in the lyophilized form. The lyophilized cultures were revived in nutrient broth and cultured on nutrient agar plates. After 24 h of incubation at 28±2°C, the bacteria were resuspended in nutrient broth for the isolation of genomic DNA.

Test sample

Clarius batracus (10-12 cm in length) were collected from local pond and kept in aquarium of 50 L capacity in 1:1 ratio of tap water: pond water. Fish were challenged by adding *P. fluorescens* broth in aquarium water (approximately 1:100 ratio). Mucus, kidney and water samples were collected after 24 h and stored at -20°C. Further genomic DNA was isolated from these samples.

Genomic DNA extraction from bacterial broth, pond water and infected tissue

Genomic DNA was isolated from five bacterial strains (*P. fluorescens, A. hydrophila*, *L. acidophilus*, *S. aureus* and *B. cereus*) grown in nutrient broth overnight, pond water, mucus and kidney tissue (isolated from infected fish crushed in liquid nitrogen) using Qiagen DNA extraction kit (Gentra Puregene protocol, 2010) according to the manufacturer's instructions.

Design of oligonucleotide primers for LAMP method

Pseudomonas sp. strains high in proteolytic activity in milk are responsible for bacterial deterioration of dairy products. Protease digestion of milk can lead to clotting and gelation of milk casein. So, a rapid test for detection of *Pseudomonas* strains in milk is required because conventional plate-counting procedure to detect psychrotrophic contamination in milk products is time-consuming and not useful to prevent food degradation. The *aprX* gene encoding an alkaline metalloprotease is considered the responsible agent for milk spoilage. LAMP primers targeting for this sequence can accelerate the detection process. Hence, DNA sequence of *P. fluorescens* Apr X gene for alkaline metalloprotease, was retrieved (partial sequence) from NCBI (http://www.ncbi:nlm.nhi.gov/) and used for primer designing. The specific LAMP DNA oligonucleotide primers were designed by using Eiken Genome site via free online software, that is, Primer-Explorer IV software program (http://loopamp.eiken.co.jp/e/lamp/). Four sets of primers (described in detail below) based on the following six distinct regions of the target gene: the F3c, F2c and F1c regions at the 3' side and the B1, B2 and B3 regions at the 5' side were designed (Table 1) and got synthesized from Sigma Aldrich.

Optimization of LAMP protocol

Bacterial DNA of *P. fluorescens* was amplified by using the following composition of LAMP reaction mixture: 7.5 µl 1x Thermopol buffer [10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2.0 mM $MgSO_4$, 0.1% Triton X-100; New England Biolab], 4 µl Betaine (0.8 M Sigma), 1.0 µl dNTPs (1.0 mM; Sigma), 0.5 µl each of F_3 and B_3 (0.2 mM; Sigma), 4.0 µl each of FIP and BIP (1.6 mM; Sigma), 1.0 µl *Bst* DNA polymerase (8U; New England Biolab), 0.5 µl nuclease free water and 2.0 µl DNA template. The reaction was carried out at different temperatures ranging from 60 to 65°C for 1 h followed by heat inactivation step at 80°C for 5 min in Accublock digital dry bath (Labnet International Inc.). The samples were kept in ice for 10 min.

Table 1. Details of primers used for LAMP assay.

S/N	Oligo name	5'<-----Sequence----->3'	Length
1	F3	TCGAGCACCATGAACAAACA	20
2	B3	GGCAGGTAAGCGAAGGC	17
3	FIP	AGGATTGCATGGCCAGTGCGCCGGGTTCAGCCAGTTC	37
4	BIP	CGGACGTGGCCAACGTGACGCTGTAGTTGCCGAAAGTC	38

Figure 1. Agarose gel illustrating the amplification of the designed primers to the *P. fluorescens* DNA. The reaction was carried out at 61°C using the 4 primer set for 1 h followed by heat inactivation step at 80°C for 5 to 7 min. Lanes: 1, negative control; 2, empty; 3, *P. fluorescens* DNA showing good amplification (ladder like band pattern).

Visualization of LAMP product

The amplified products were analyzed in 2.0% agarose gel in Tris Borate EDTA (1x) buffer stained with ethidium bromide and photographed using the Bio Red gel documentation and analysis system (XR).

Specificity test

The ability of LAMP assay developed for detection of *P. fluorescens* specifically was evaluated by using different bacterial DNA *viz. P. fluorescens*, *L. acidophilus*, *A. hydrophila, S. aureus* and *B. cereus*. The specificity of the LAMP was determined on the basis of DNA amplification.

Sensitivity test

DNA of *P. fluorescens* was serially diluted up to 10^{-10} dilutions with nuclease free water and lower detection limit was tested.

Detection of *P. fluorescens* in broth, pond water, infected tissue and mucus samples

Bacterial broth, pond water, mucus and infected crushed kidney tissue samples of fish were used to check the potential of LAMP method to amplify nucleic acid directly from live cells of *P. fluorescens*. Finally, LAMP assay was performed with these test samples under optimized conditions.

RESULTS AND DISCUSSION

LAMP method successfully detected *P. fluorescens* DNA as ladder like pattern on gel after isothermal amplification by *Bst polymerase*.

Optimization of LAMP reaction conditions for *P. fluorescens*

P. fluorescens DNA was tested under different conditions of temperature and time, that is, 60 to 65°C for 1 h followed by heat inactivation step at 80°C for 5 to 10 min in order to determine best amplification reaction conditions. Results show that optimum temperature and time for amplification of *P. fluorescens* DNA was found to be 61°C for 60 min and 80°C for 5 to 7 min (Figure 1) and no amplification was observed in negative control. While at other temperatures, very poor (smear like band pattern at 63°C) or no amplification was observed (Gahlawat et al., 2009). Fernandez-Soto et al. (2014) detected *Schistosoma mansoni* by using *Loopamp* DNA amplification Kit tested with the *S. mansoni* primer set and found that LAMP reaction successfully take place at temperatures of 61, 63 and 65°C within the temperature range (60-65°C). Similarly, Uemura et al. (2008) detected *Pneumocystis pneumonia* by using LAMP assay at 61°C temperature.

Specificity of LAMP assay for *P. fluorescens*

DNA samples of *P. fluorescens*, *L. acidophilus*, *A. hydrophila, S. aureus* and *B. cereus* were tested for

Figure 2. Agarose gel illustrating the direct detection of bacteria from collected field samples infected with *P. fluorescens* by LAMP method. The reaction was carried out at 61°C using the 4 primer set for 1 h. Lanes: 1, DNA ladder (100 bp); 2, negative control; 3, positive control (*P. fluorescens* DNA); 4, kidney tissue; 5: DNA isolated from infected tissue; 6, pond water sample; 7, DNA isolated from infected pond water; 8, DNA isolated from test water infected with mixture of pathogenic bacteria.

specificity of the LAMP product. LAMP assay specifically amplified only sequence of the target, that is, DNA of *P. fluorescens* only while no amplification was observed with DNA samples from other bacterial strains (*B. cereus*, *A. hydrophila, S. aureus* and *L.acidophilus*) (Figure not shown). Also, LAMP assay effectively detected only *P. fluorescens* DNA from mixture of DNA isolated from test water infected with mixture of pathogenic bacteria (Figures 2 Lane: 8). Specificity of LAMP method was justified by use of four different primers for recognizing six distinct sequences on target gene. LAMP primers were found to be highly specific to the Apr X gene for alkaline metalloprotease because they did not attach to other bacterial DNA sequences. The specificity of LAMP assay was observed by the absence of any cross reaction with other tested bacterial strains indicating a high specificity when among 116 reference strains, only methicillin-resistant *S. aureus* was detected by *orfX*-LAMP assay (Su et al., 2014).

Sensitivity limit of LAMP

The detection limit of LAMP assay was tested with serially diluted DNA samples of *P. fluorescens*. Samples were tested up to 10^{-10} dilution and last detection limit of LAMP was found to be approximately 10 pg according to dilution (Figure not shown). Similar sensitivity was reported by Pan et al. (2011) in *Brucella* species.

In sensitivity evaluations, Hui et al. (2013) successfully detected a serial dilution of extracted *Bordetella bronchiseptica* DNA with a detection limit of 9 copies, which was 10 times more sensitive than that of PCR. He also considered that there is no need for the complex instrumentation making this LAMP assays a promising alternative for the diagnosis of *B. bronchiseptica* in rural areas and developing countries where there is lack of complex laboratory services.

Direct detection of *P. fluorescens* from bacterial broth, pond water, tissue and mucus

LAMP assay was also found an to be an effective tool for the detection of *P. fluorescens* bacteria directly from field samples, that is, pond water and infected kidney tissue of fish (Figure 2). Although there are very few reports related to direct detection of bacteria from broth, water as well as from infected tissue by using LAMP method (Iwamoto et al., 2003; Savan et al., 2004; Poon et al., 2006; Kubota et al., 2008; Gahlawat et al., 2009) but still, this method may prove useful for direct detection of bacteria from the sites of infection and thus can be helpful in field testing. However, this method can detect *P. florescens* DNA from kidney tissue easily (Figure 2). So, LAMP can directly detect the bacteria from pond water and tissue samples and thus, it can be used as valuable tool for easy and robust on-the-spot detection of this bacterium in clinical laboratories and field conditions.

Conflict of interests

The authors did not declare any conflict of interest.

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REFERENCES

Aly S (2001).Light and electron microscopic studies on pseudomoniasis among common carp (*Cyprinus carpio*).Suez Canal Vet. Med. J. 4: 95 -103.

Aly S, El-Attar A, El-Genedy H (2002). Role of fish in transmission of

Pseudomonas fluorescens to ducklings with a trial for treatment and control. Pathologic & Electron Microscopic Examinations,10th Scientific Vet. Med. Conference, Assuit Univ.17 -19 December, 187-208.

CDC (2005). *Pseudomonas* bloodstream infections associated with a heparin/saline flush-Missouri, New York, Texas, and Michigan, 2004-2005. Morb. Mortal. Wkly Rep. 54: 269-272.

Chen J, Ma X, Yuan Y, Zhang W (2011).Sensitive and rapid detection of *Alicyclobacillus acidoterrestris* using loop-mediated isothermal amplification. J. Sci. Food Agric. 9:1070.

Eissa NME, Abou El-Ghiet EN, Shaheen AA, Abbass A (2010). Characterization of *Pseudomonas* species Isolated from Tilapia "*Oreochromis niloticus*" in Qaroun and Wadi-El-Rayan Lakes, Egypt Glob. Vet. 5 (2): 116-121.

El-Hady MA, Samy AA (2011). Molecular typing of *Pseudomonas* species isolated from some cultured fishes in Egypt.Global Veterinaria. 7 (6): 576-580.

Feldman M, Bryan R, Rajan S, Scheffler L, Brunnert S, Tang H, Prince A (1998).Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. Infect. Immun. 66(1):43-51.

Fernandez-Soto P, Gandasegui Arahuetes J, Sanchez Hernandez A, Lopez Aban J, Vicente Santiago B, Muro A (2014). A Loop-Mediated Isothermal Amplification (LAMP) Assay for Early Detection of *Schistosoma mansoni* in Stool Samples: A Diagnostic Approach in a Murine Model. PLoS Negl. Trop. Dis. 8(9): e3126.

Gahlawat SK, Ellis AE, Collet B (2009).A sensitive loop-mediated isothermal amplification (LAMP) method for detection of *Renibacterium salmoninarum*, causative agent of bacterial kidney disease in Salmonids. J. Fish Dis. 32:491.

Han HJ, Jung SJ, Oh MJ, Kim DH (2011).Rapid and sensitive detection of *Streptococcus iniae* by loop-mediated isothermal amplification (LAMP). J. Fish Dis. 34:395.

Hirvonen MR, Huttunen K, Roponen M (2005). Bacterial strains from moldy buildings are highly potent inducers of inflammatory and cytotoxic effects. Indoor Air 15(9):65-70.

Hsueh PR, Teng LJ, Pan HJ, Chen YC, Sun CC, Ho SW, Luh KT (1998).Outbreak of *Pseudomonas fluorescens* bacteremia among oncology patients. J. Clin. Microbiol. 36:2914-2917.

http://loopamp.eiken.co.jp/e/lamp/

http://www.ncbi:nlm.nhi.gov/

Hui Z, Zhu L, Zhou Y, Ji H, Dai H, Guo W, Xu Z (2013). Rapid and sensitive detection of *Bordetella bronchiseptica* by loop-mediated isothermal amplification (LAMP). Pesqui. Vet. Bras. 33(10):1222-1226.

Huttunen K, Hyvarinen A, Nevalainen A, Komulainen H, Hirvonen MR (2003).Production of proinflammatory mediators by indoor air bacteria and fungal spores in mouse and human cell lines. Environ. Health Perspect. 111:85-92.

Ikadai H, Tanaka H, Shibahara N, Matsuu A, Uechi M, Itoh N, Oshiro S, Kudo N, Igarashi I, Oyamada T (2004). Molecular evidence of infections with *Babesia gibsoni* parasites in Japan and evaluation of the diagnostic potential of a loop-mediated isothermal amplification method. J. Clin. Microbiol. 42:2465.

Iseki H, Alhassan A, Ohta N, Thekisoe OM, Yokoyama N, Inoue N, Nambota A, Yasuda J, Igarashi I (2007).Development of a multiplex loop-mediated isothermal amplification (mLAMP) method for the simultaneous detection of bovine *Babesia* parasites. J. Microbiol. Methods 71:28.

Iwamoto T, Sonobe T, Hayashi K (2003). Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium* and *M. intracellulare* in sputum samples. J. Clin. Microbiol. 41:2616.

Kubota R, Vine BG, Alvarez AM, Jenkins DM (2008).Detection of *Ralstonia solanacearum* by loop-mediated isothermal amplification. Phytopathology 98:1045.

Liu PV, Mercer CB (1963).Growth, toxigenicity and virulence of *Pseudomonas Aeruginosa*. J. Hygiene 61: 485-491.

Lu Q, Gerrits van den Ende AH, Bakkers JM, Sun J, Lackner M, Najafzadeh MJ, Melchers WJ, Li R, de Hoog GS (2011).Identification of *Pseudallescheria* and *Scedosporium* species by three molecular methods. J. Clin. Microbiol. 49:960.

Masbouba IM (2004). Studies on *Pseudomonas* Infection in fish in Kafr El - Sheikh Province. Unpublished M V Sc.Thesis, Tanta University.

Nagamine K, Hase T, Notomi T (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell. Probes.16: 223.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000).Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28: E63.

Pan W, Wang JY, Shen HY, Zhao MG, Ju CM, Dong XY, Lin YI L, Chen DJ (2011). Development and application of the novel visual loop-mediated isothermal amplification of omp25 sequence for rapid detection of *Brucella* sp. J. Anim. Vet. Adv. 10 (16): 2120-2126.

Pittman M (1953). A study of bacteria implicated in transfusion reactions and of bacteria isolated from blood products. J. Lab. Clin. Med. 42(2):273-88.

Poon LL, Wong BW, Ma EH, Chan KH, Chow LM, Abeyewickreme W, Tangpukdee N, Yuen KY, Guan Y, Looareesuwan S, Peiris JS (2006). Sensitive and inexpensive molecular test for falciparum malaria: detecting *Plasmodium falciparum* DNA directly from heat-treated blood by loop-mediated isothermal amplification. Clin. Chem. 52(2):303.

Roberts RJ (1978).Fish Pathology, 2nd ed. Bailliere Tindall, London.

Saharan P, Dingolia S, Khatri P, Duhan JS, Gahlawat SK (2014a).Loop-mediated isothermal amplification (LAMP) based detection of bacteria: A Review. Afr. J. Biotechnol. 13 (19):1920-1928.

Saharan P, Khatri P, Dingolia S, Duhan JS, Gahlawat SK (2014b). Rapid detection of viruses using loop-mediated isothermal amplification (LAMP) A Review, In: Biotechnology: Prospects and Applications, edited by RK Salar, SK Gahlawat, P Siwach and JS Duhan (Springer, New Delhi, Heidelberg, New York, Dordrecht, London) 2014, 287. DOI 10.1007/978-81-322-1683-4.

Savan R, Igarashi A, Matsuoka S, Sakai M (2004).Sensitive and rapid detection of Edwardsiellosis in fish by a loop-mediated isothermal amplification method. Appl. Environ. Microbiol. 70(1):621.

Scott J, Boulton FE, Govan JR, Miles RS, McClelland DB, Prowse CV (1988).A fatal transfusion reaction associated with blood contaminated with *Pseudomonas fluorescens.* Vox Sang. 54:201-204.

Somsiri T, Soontornvit S (2002). Bacterial diseases of cultured tiger frog (*Rana tigerina*). In C.R. Lavilla-Pitogo and E.R. Cruz-Lacierda (eds.), Diseases in Asian Aquaculture IV, Fish Health Section, Asian Fisheries Society, Manila.

Su J, Liu X, Cui H, Li Y, Chen D, Li Y, Yu G (2014).Rapid and simple detection of methicillin-resistance *Staphylococcus aureus* by *orfX* loop-mediated isothermal amplification assay. BMC Biotechnol. 14:8.

Sutter VL (1968). Identification of *Pseudomonas* species isolated from hospital environment and human sources. Appl. Microbiol. 16:1532-1538.

Uemura N, Makimura K, Onozaki M, Otsuka Y, Shibuya Y, Yazaki H, Kikuchi Y, Abe S, Kudoh S (2008).Development of a loop-mediated isothermal amplification method for diagnosing *Pneumocystis pneumonia.* J. Med. Microbiol. 57(1):50.

Wang Y, Kang Z, Gao H, Gao Y, Qin L, Lin H., Yu, F, Qi X, Wang X (2011). A one-step reverse transcription loop-mediated isothermal amplification for detection and discrimination of infectious bursal disease virus. Virol. J. 8:108.

Weyant RS, Moss CW, Weaver RE, Hollis DG, Jordan JG, Cook EC, Daneshvar MI (1996).Identification of unusual pathogenic gram-negative aerobic and facultatively anaerobic bacteria. 2nd ed. Baltimore, MD: Williams and Wilkins.

Zhao K, Shi W, Han F, Xu Y, Zhu L, Zou Y, Wu X, Zhu H, Tan F, Tao S, Tang X (2011). Specific, simple and rapid detection of porcine *circovirus* type 2 using the loop-mediated isothermal amplification method. Virol. J. 8:126.

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Full Length Research Paper

Detection of novel polymorphisms in the mitochondrial DNA D-Loop hypervariable region HVI from 400 healthy unrelated individuals from central and North-central Iraq

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The mitochondrial DNA (mtDNA) is a small circular genome located within the mitochondria in the cytoplasm of the cell and a smaller 1.2 kilobase pair fragment, called the control region (D-loop). The aims of this research were to study this region by using the Sanger sequencing technique and establish the degree of variation characteristic of a fragment. FTA® Technology (FTA™ paper DNA extraction) was utilized to extract DNA. PCR products were purified by EZ-10 spin column then sequenced and detected by using the ABI 3730xL DNA Analyzer. Novel polymorphisms discovered at positions 16037, 16075, 16104 and 16201 in future may be suitable sources for identification purpose.

Key words: D-loop, frequency, north-central Iraq, mitochondrial DNA, polymorphism.

INTRODUCTION

Mitochondrial DNA (mtDNA) is a useful genetic marker for answering evolutionary questions due to its high copy number, maternal mode of inheritance, and its high rate of evolution (Stoneking and Soodyall, 1996; Kraytsberg et al., 2004; Imad et al., 2015a). mtDNA is a small circular genome located within the mitochondria in the cytoplasm of the cell. The mitochondrial genome can be divided into two regions: a large coding region, which is responsible for the production of various biological molecules involved in the process of energy production in the cell, and a smaller 1.2 kilobase pair fragment, called the control region. It is found to be highly polymorphic and harbors three hypervariable regions (HV), HV1, HV2 and HV3 (Helgason et al., 2003; Ingman and Gyllensten, 2003; Ukhee et al., 2005). mtDNA molecules contain 37 genes coding for 22 tRNAs, two rRNAs and 13 mRNAs (Brown et al., 1993; Giulietta et al., 2000; Young, 2009; Imad et al., 2014a). In modern population genetics research, studies based on mtDNA and Y-chromosome DNA are an excellent way of illustrating population structure while tracing uni-parental inheritance and ancestry since mtDNA is maternally inherited while the Y-chromosome is paternally inherited.

mtDNA is therefore inherited from generation to

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Table 1. Primer sequence, region amplified, fragment size (bp) and PCR product length for HVI.

Primer name	Primer sequence (5' - 3') (Forward; F, Reverse; R)	Region amplified	Fragment size (bp)	PCR Product length
HVI-1	F: 5'- TCGTACATTACTGCCAGCCA -3'	16094-16113	20	103
	R: 5'- CATGGGGAGGGGGTTTTGAT -3'	16196-16177	20	
HVI-2	F: 5'- AAACCCCCTCCCCATGCTTA -3'	16181-16200	20	117
	R: 5'- AGGGTGGGTAGGTTTGTTGG -3'	16297-16278	20	
HVI-3	F: 5'-TCACCCATCAACAACCGCTA -3'	16068-16087	20	133
	R: 5'- TAAGCATGGGGAGGGGGTTT -3'	16200-16181	20	
HVI-4	F: 5'-TTCGTACATTACTGCCAGCCA-3'	16093-16113	20	100
	R: 5'- GGGAGGGGGTTTTGATGTGG -3'	16192-16173	20	
HVI-5	F: 5'-TCATGGGGAAGCAGATTTGGG-3'	16029-16049	21	91
	R: 5'- TCATGGTGGCTGGCAGTAAT -3'	16119-16100	20	
	R: 5'-GTCTGTGTGGAAAGTGGCTGT-3'	277-257	21	

generation through the maternal line. Mechanisms for this include simple dilution (an egg contains 100,000 to 1,000,000 mtDNA molecules, whereas a sperm contains only 100 to 1000), degradation of sperm mtDNA in the fertilized egg, and at least in a few organisms, failure of sperm mtDNA to enter the egg. Most mitochondria are present at the base of the sperm's tail, which is used for propelling the sperm cells; sometimes the tail is lost during fertilization. Also, unlike nuclear DNA, where there is a shuffling of the chromosomes at every generation, the mitochondrial DNA does not recombine with any other DNA type and remains intact from generation to generation (Brown, 2000; Brown, 2002; Dobbs et al., 2002; Imad et al., 2014b). mtDNA from all mammalian is very similar, with the order and position of the genes being the same.

Genetic analyses in population studies of the mitochondrial genome can be done either by sequencing the mtDNA or through the use of restriction fragment length polymorphisms (RFLPs) (Guntheroth et al., 1986; Pastore, 1994). RFLPs utilize restriction enzymes that can recognize the presence or absence of specific polymorphic DNA regions, and cut sites in the coding region of the mtDNA. These polymorphisms allow scientists to compare mtDNA from crime scenes to mtDNA from given individuals to ascertain whether the tested individuals are within the maternal line (or another coincidentally matching maternal line) of people who could have been the source of the trace evidence.

MATERIALS AND METHODS

Sample collection

Population sample collected from 400 healthy unrelated volunteer donors, recruited from central and north-central Iraq, where the samples were collected from donors after they have been displaced to the center of Iraq because of the bad security situation in the provinces at north of the center stricken.

mtDNA extraction and amplification

DNA was extracted from all dried blood samples on FTA cards following the manufacture's procedure as described in Whatman FTA Protocol BD01 except that the Whatman FTA purification reagent was modified to half the volume reference (Imad et al., 2014c). A 1.2 mm diameter disc was punched from each FTA card with a puncher. The discs were transferred to new Eppendorf tubes and washed three times in 100 µl Whatman FTA purification reagent. Each time, the disc was incubated for 5 min at room temperature with moderate manual mixing and the reagent was discarded between washing steps. The discs were then washed twice in 200 µl TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), the buffer was discarded and the discs were left to dry at room temperature for 1 h. Amplification of HVI region was carried out using five sets of primers (Table 1). A portion of a noncoding region encompassing positions from 16024 to 16365 for HVI was amplified in accordance with the Anderson reference sequence (Anderson et al., 1981). 20 µL of Master Mix was added into PCR tube. Change the pipette tip and add 20 µL of Primer Mix into PCR tube. Add 10 µL of extracting DNA into the PCR tube after changing the pipette tip again. Allow all the liquid settles at the bottom of the tube, and not elsewhere. Check the volume in the PCR tube using the PCR tube with 50 µL in it. The location of the tube on the grid after putting the mixture in the thermal cycler 95°C hold for 10 min, 30 cycles of 94°C for 30 s, 52.5°C for 30 s, 65°C for 1 min, 72°C hold for 10 min, 4°C hold ∞ infinity which is the cycling protocol for amplification of mtDNA PCR.

Purification, cycle sequencing and sequence analysis of mitochondrial DNA

mtDNA PCR products were purified by EZ10-spin column DNA clean up kit 100 prep. The DNA sequencing of the PCR products

was done using the BigDye TM Terminator and utilizing POP-7 polymer (Applied Biosystems). The separation of the cycle sequencing products was carried out. Detection was by using the ABI 3730xL DNA Analyzer, cap array size of 96 and cap array length of 50. The reference sequence described by Anderson et al. (1981) was compared to the data observed. Within the mtDNA coding region, sequencing results were studied from a consensus sequence derived from multiple sequence results. Data were analysed by Sequencher™ and aligned with the Anderson sequence (Anderson et al., 1981) using sequence Navigator software.

Statistical analysis

Genetic diversity for the analyzed DNA fragment was calculated according to the formula:

$$h=(1-\sum x_i^2)n/(n-1)$$

Where, n is sample size and x_i is the frequency of i-th mtDNA type (Gu, 2001).

The probability of two randomly selected individuals from a population having identical mtDNA types is:

$$(P=\sum x_i^2)$$

Where, p frequencies of the observed Haplotypes (Jones, 1972).

RESULTS AND DISCUSSION

The basic aim of this work was to assess the degree of variation characterizing a selected segment of the non-coding region of mtDNA. The study enabled identification of 117 different haplotypes and 30 polymorphic nucleotide positions (Table 2). Within these 30 variations, the most frequent variant (H1) was consistent with the Anderson sequence, transitions between T and C, transitions between A and G and only and transversions. This fact is consistent with abundant literature data revealing significant domination of transitions over transversions (Brown et al., 1982; Yang and Yoder, 1999; Imad et al., 2015b).

Eight polymorphic positions (16037, 16041, 16067, 16075, 16104, 16144, 16201 and 16234) showed trans-versions (Table 2) and 4 are novel (Table 3); the number of analyzed markers was been increased to compensate for the increasing number of profiles in the databases in order to minimize accidental matches between unrelated individuals. Progression of new technology is therefore very slow and the use of SNPs has sometimes met a reluctant reception.

Genetic diversity for the analysed DNA fragment was calculated according to the formula: D= 1-$\sum p^2$ and recorded 95.4%. The calculated value of the genetic diversity should be understood as high in the context of noncoding function of the analysed DNA fragment. The relatively high gene diversity and a relatively low random match probability were observed in this study.

Comparative analysis of our results with previously published Iraqi data revealed significant differences in varying patterns (Nadia et al., 1999). This observation supports the hypothesis that different SNP-type polymorphisms can be strongly associated with a given population. Haplotypes detected in this study group have been compared with other global populations: German (n = 200) (Lutz et al., 1998), US Caucasian (n = 604), Africa (n = 111), Malaysia (n = 195) (Budowle et al., 1999) and India (n = 98) (Mountain et al., 1995) (Table 4).

Walsh et al. (1991) and Tang (2002) showed that the polymorphisms of mtDNA coding area are less than that of mtDNA control region. Therefore, more efficient polymorphic sites should be used to provide an improved discrimination power for forensic mtDNA testing (Imad et al., 2014). As forensic markers, they should be phenotypically neutral to avoid landing investigators into serious situations of medical genetic privacy and ethnics, especially for mtDNA coding area whose mutation is often correlated with an increased risk of some disease. With the whole mtDNA sequences being researched, we are optimistic that the polymorphisms sites within mtDNA coding area will be useful in combination with SNPs in the control region in order to increase the discrimination power of mtDNA (David et al., 2013; Imad et al., 2014).

Conclusion

Sequence analysis of the noncoding region of mtDNA (HVI) conducted on a population of 400 unrelated individuals enabled identification of 117 different haplotypes. The novel polymorphic positions 16037, 16075, 16104 and 16201 described in future may be suitable sources for identification purpose.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Table 2. Variable positions and haplotypes for HVI.

Anderson	16037	16038	16041	16048	16063	16067	16069	16071	16075	16083	16092	16104	16124	16140	16144	16145	16147	16148	16153	16162	16163	16171	16179	16183	16201	16209	16217	16224	16234	16247	No. of Individual
	A	A	A	G	T	C	C	C	T	C	T	C	T	T	T	G	C	C	G	A	A	A	C	A	C	T	T	T	C	A	
H1*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	210
H2	G		-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	1
H3	-	G	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	G	5
H4	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	C	-	-	-	1
H5	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	G	-	-	-	-	-	-	1
H6	G	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	1
H7	-	-	G	-	-	-	-	-	-	-	-	-	C	-	-	-	-	T	-	-	-	-	-	G	-	-	-	-	-	-	1
H8	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	C	-	-	1
H9	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	1
H10	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	G	-	-	-	-	-	-	-	-	3
H11	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	2
H12	G	-	-	A	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	1
H13	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	1
H14	-	-	-	-	-	T	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	1
H16	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	T	-	-	-	-	-	2
H17	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	2
H18	-	-	G	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H19	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H20	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
H21	-	-	-	-	-	-	T	-	C	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	1
H22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	1
H23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	T	-	3
H24	-	-	-	-	-	-	-	-	-	T	C	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H25	T	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	1
H26	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H27	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H28	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	T	-	-	-	-	-	4
H29	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	2
H30	-	-	-	A	-	-	-	-	C	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	G	2
H31	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H32	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	1
H33	-	-	-	-	C	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H34	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	1

Table 2. Contd.

H35	-	-	G	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	6
H36	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	1
H37	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	C	-	-	-	2
H38	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	C	-	-	-	1
H39	-	-	-	-	-		-	-	-	-	-	-	C	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	1
H40	-	-	-	-	-		T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H41	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	1
H42	G	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	5
H43	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4
H44	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	3
H45	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	T	C	-	-	-	-	3
H46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
H47	-	-	-	-	-	-	-		C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	T	-	3
H48	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	1
H49	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	1
H51	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	1
H52	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H53	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	C	-	-	1
H54	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	1
H55	G	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6
H56	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	T	-	-	-	-	-	T	-	-	-	-	-	-	-	1
H57	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T		1
H58	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		G	1
H59	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	T	-	-	-	-	-			1
H60	-	-	G	-	-	-	-	-	-	-	-	-	C	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H61	-	G	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	1
H62	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	C	-	-	-	1
H63	-	-	-	A	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	1
H64	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	G	-	1
H65	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	2
H66	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	2
H67	-	-	-	-	-	-	T	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	2
H68	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H69	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	G	-	-	-	-	-	-	2
H70	-	-	-	A	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H71	-	-	-	-	-	T	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	3

Table 2. Contd.

H72	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	1
H73	-	-	-	-	-	-	-	T	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H74	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	1
H75	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	1
H76	-	-	-	-	C	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	1
H77	-	-	G	-	-	-	-	-	-	-	-	-	-	C	-	-	T	-	-	-	-	-	T	-	-	-	-	-	-	-	1
H78	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
H79	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	G	-	G	-	-	-	-	-	-	-	-	2
H80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	2
H81	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	C	-	-	-	4
H82	-	-	-	A	-	-	-	-	-	-	C	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H83	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	1
H84	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H85	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	G	1
H86	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	1
H87	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	T	-	-	-	-	-	1
H88	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	C	-	-	-	-	1
H89	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H90	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	6
H91	-	-	-	-	C	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H92	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	C	-	-	1
H93	-	-	C	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	1
H94	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	1
H95	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	G	-	-	-	-	-	-	-	-	1
H96	-	-	-	-	-	-	-	T	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	1
H97	-	-	-	A	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4
H98	-	-	-	-	C	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	3
H99	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	C	-	G	2
H100	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	T	-	-	-	-	-	-	-	-	-	-	-	-	2
H101	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-		-	-	-	-	-	G	-	-	-	-	-	-	-	-	2
H102	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	T	-	-	-	-	-	-	-	1
H103	-	-	-	-	-	-	-	-	-	-	C	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H104	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	1
H105	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	1
H106	-	-	-	-	-	-	-	-	-	-	-	-	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H107	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	1
H108	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	C	-	-	1

Table 2. Contd

H109	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	1
H110	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	1
H111	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	1
H3112	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	C	-	-	-	1
H113	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H114	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	1
H115	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	C	-	-	1
H116	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	2
H117	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	C	-	-	-	1

Table 3. Types of mutations in variable positions for HVI.

Position	Mutation	Type of mutation	Presence in Mitomap	Frequency	Frequency %
16037	Transition	A-G	Presence	0.0425	4.25
16037	Transversion	A-T	New*		
16038	Transition	A-G	Presence	0.0425	4.25
16041	Transition	A-G	Presence	0.0375	3.75
16048	Transition	G-A	Presence	0.0525	5.25
16063	Transition	T-C	Presence	0.0555	5.55
16067	Transition	C-T	Presence	0.0325	3.25
16069	Transition	C-T	Presence	0.0375	3.75
16071	Transition	C-T	Presence	0.04	4.00
16075	Transition	T-C	Presence	0.0325	3.25
16075	Transversion	T-A	New*		
16083	Transition	C-T	Presence	0.03	3.00
16092	Transition	T-C	Presence	0.03	3.00
16104	Transition	C-T	Presence	0.02	2.00
16104	Transversion	C-G	New*		
16124	Transition	T-C	Presence	0.035	3.50
16140	Transition	T-C	Presence	0.0425	4.25
16144	Transition	T-C	Presence	0.0325	3.25
16144	Transversion	T-A	Presence		
16145	Transition	G-A	Presence	0.0455	4.55
16147	Transition	C-T	Presence	0.0325	3.25
16148	Transition	C-T	Presence	0.0575	5.75
16153	Transition	G-A	Presence	0.04	4.00

Table 3. Contd.

16162	Transition	A-G	Presence	0.0275	2.75
16163	Transition	A-G	Presence	0.0125	1.25
16171	Transition	A-G	Presence	0.03	3.00
16179	Transition	C-T	Presence	0.0475	4.75
16183	Transition	A-G	Presence	0.025	2.50
16201	Transition	C-T	Presence	0.0425	4.25
16201	Transversion	C-G	New*		
16209	Transition	T-C	Presence	0.0275	2.75
16217	Transition	T-C	Presence	0.0425	4.25
16224	Transition	T-C	Presence	0.035	3.50
16234	Transition	T-C	Presence	0.0305	3.10
16247	Transition	T-C	Presence	0.035	3.50
Genetic diversity*	$D= 1-\sum p^2 = 0.954$		= 95.4 %		

New*: new polymorphic positions; Genetic diversity*, Genetic diversity for the analysed DNA fragment was calculated according to the formula: $D= 1-\sum p^2$.

Table 4. Comparisons of the characteristics across D-loop region in different human population groups.

Population	Iraq[1]	India[2]	Malaysia[3]	Africa[4]	German[5]	US Caucasian[6]
Sample size	400	98	195	111	200	604
No. of variant sites	30	83	149	97	153	233
A→G	104	233	473	323	330	1112
G→A	155	66	81	78	55	219
T→C	110	145	461	382	308	1007
C→T	115	117	321	486	199	688
A→T	2	1	2	0	4	2
A→C	0	23	81	15	5	47
G→T	0	0	0	18	0	1
G→C	0	0	3	0	1	6
C→A	0	0	30	17	11	12
C→G	9	4	1	6	19	6
T→A	2	7	5	0	1	0
T→G	0	0	3	0	0	0
Insertion	0	168	322	140	291	983
Deletion	0	0	28	6	6	14

Note: % of transitions and transversions were calculated as number of observations divided by total substitutions.[1]This study; [2]Mountain et al. (1995); [3]Budowle et al. (1999); [4]Budowle et al. (1999); [5]Lutz et al. (1998); [6]Budowle et al. (1999).

REFERENCES

Anderson S, Bankier AT, Barrell BG (1981). Sequence and organization of the human mitochondrial genome. Nature 290:457-465.

Brown JR, Bechenbach AT, Smith MJ (1993). Intraspecific DNA sequence variation of the mitochondrial control region of white sturgeon (Acipenser transmontanus). Mol. Biol. Evol. 10:326-341.

Brown MD (2000). The role of MtDNA background in disease expression: Anew primary LHON mutation associated with Western Eurasian haplogroup. J. Hum. Genet. 110(2):130-138.

Brown MD (2002). Functional analysis of lymphoplast and cybrid mitochonderia containing the 3460. 11778. or 14484 Lebersheredity optic neuropathy mitochonderial DNA mutation. J. Biol. Chem. 275(51):39831-39836.

Brown WM, Prager EM, Wang A (1982). Mitochondrial DNA sequences of primates: tempo and mode of evolution. J. Mol. Evol. 18:225-239.

Budowle B, Wilson MR, DiZinno JA (1999). Mitochondrial DNA regions HVI and HVII population data. Forensic Sci. Int. 103:23-35.

David HW, Jonathan K, Arthur JE (2013). Validation of the PLEX-IDTM mass spectrometry mitochondrial DNA assay. Int. J. Legal Med. 127(2):277-286.

Dobbs LJ, Madigan MN, Carter AB (2002). Use of FTA gene guard filter paper for the storage and transportation of tumor cells for molecular testing. Arch. Pathol. Lab Med. 126 (1):56-63.

Giulietta DB, Ivane SN, Michele S (2000). Mitochondrial DNA sequences in prehistoric human remains from the Alps. Eur. J. Hum. Genet. 8:669-677.

Gu MB (2001). Polymorphism study of the mitochondrial DNA control region in Chinese Han population group. Chin. J. Forensic Med. 16(1):6-9.

Guntheroth WG, Lohmann R, Spiers PS (1986). Risk of sudden infant death syndrome in subsequent sibling. J. Pediatr. 116(4):520-524.

Helgason A, Hrafnkelsson B, Gulcher JR (2003). A populationwide coalescent analysis of Icelandic matrilineal and patrilineal genealogies: evidence for a faster evolutionary rate of mtDNA lineages than Y chromosomes. Am. J. Hum. Genet. 72:1370-1388.

Imad H, Mohammed A, Cheah Y (2014a). Genetic variation of twenty autosomal STR loci and evaluate the importance of these loci for forensic genetic purposes. Afr. J. Biotechnol. 13:1-9.

Imad H, Muhanned A, Aamera J, Cheah Y (2014c). Analysis of eleven Y-chromosomal STR markers in middle and South of Iraq. Afr. J. Biotechnol. 13(38):3860-3871.

Imad HH, Ameer IA, Mohammed AJ (2014b). Haplotypes and variable position detection in the mitochondrial DNA coding region encompassing nucleotide positions 10,716-11,184. Mitochon. DNA. 1-6.

Imad HH, Mohammed AJ, Muhanned AK (2015a). Forensic analysis of mitochondrial DNA hypervariable region HVII (encompassing nucleotide positions 37 to 340) and HVIII (encompassing nucleotide positions 438-574) and evaluate the importance of these variable positions for forensic genetic purposes. Afr. J. Biotechnol. 14(5):365-375.

Imad HH, Muhanned AK, Rafid HH (2015b). X-chromosome short tandem repeat, advantages and typing technology review. African J. of Biotechnology. 14(7): 535-541.

Ingman M, Gyllensten U (2003). Mitochondrial genome variation and evolutionary history of Australian and New Guinean Aborigines. Genome Res. 13(7):1600-1606.

Jones DA (1972). Blood samples: probability of discrimination. J. Forensic Sci. Soc. 12:355-358.

Kraytsberg Y, Schwartz M, Brown TA (2004). Recombination of human mitochondrial DNA. Science 304(56731):28-981.

Lutz S, Weisser H-J, Heizmann J (1998). Location and frequency of polymorphic positions in the mtDNA control region of individuals from Germany". Int. J. Legal Med.111:67-77.

Mohammed A, Imad H. (2013) Autosomal STR: From locus information to next generation sequencing technology. Res. J. Biotechnol. 8(10):92-105.

Mohammed AJ, Imad HH, Muhanned AK (2015). Detection of New Variant "Off-ladder" at the (D12S391, D19S433 and D1S1656 loci) and Tri-allelic Pattern at the D16S539 Locus in a 21 Locus Autosomal Short Tandem Repeat Database of 400 Iraqi Individuals. Afr. J. of Biotechnol.. 14(5): 375-399.

Mountain JL, Hebert JM, Bhattacharyya S (1995). Demographic history of India and mtDNA-sequence diversity. Am. J. Hum. Genet. 56:979-992.

Nadia A, Maria P, Vincenza B (2011). In search of the genetic footprints of Sumerians: a survey of Y-chromosome and mtDNA variation in the Marsh Arabs of Iraq. BMC Evolut. Biol. 11: 288.

Pastore GM (1994). Leigh syndrome and hypertrophic cardiomyopathy in an infant with a mitochonderial DNA point mutation (T8993). Am. J. Med. Genet. 50: 265-71.

Stoneking M, Soodyall H (1996). Human Evolution and the Mitochondrial Genome. Curr. Opin. Genet. Dev. 6(6):731-736.

Tang H (2002). Polymorphisms of mitochondrial DNA hypervariable region of Beijing Han population. Chin. J. Forensic Med. 17(1):32- 33.

Ukhee C, Hwan YL, Ji-Eun Y (2005). Mitochondrial DNA CA dinucleotide repeats in Koreans: the presence of length heteroplasmy. Int. J. Legal Med. 119:50-53.

Walsh PS, Metzger DA, Higuchi R (1991). Chelex 100 as amedium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10(4):506-513.

Yang Z, Yoder AD (1999). Estimation of the transition/transversion rate bias and species sampling. J. Mol. Evol. 48:274-283.

Young KL (2009). The Basques in the Genetic Landscape of Europe. PhD Dissertation. University of Kansas.

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Oil palm fruit fibre promotes the yield and quality of *Lentinus squarrosulus* (Mont.) Singer, an edible Nigerian mushroom

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Agricultural production and the agro-food industry furnish large volumes of solid wastes, which when unutilized could lead to environmental pollution. An attempt was made to utilize wastes from the oil palm and timber industries for the cultivation of *Lentinus squarrosulus*, a Nigerian edible mushroom. Mahogany sawdust (MSD), *Gmelina* sawdust (GSD), oil palm fruit fibre (OPFF) and oil palm empty fruit bunch (OPEFB) significantly influenced crop cycle time, yield, nutritional properties and market quality of the mushroom. The shortest crop cycle time achieved (47 days) was with *Gmelina* sawdust. Oil palm fruit fibre proved a better substrate for the production of mushrooms with higher yields and protein content (30.10 g/kg substrate and 27.42%). Yield and protein content of harvested mushrooms were strongly correlated with the nitrogen content of the substrates. Fruit bodies with the lowest fat content were harvested from *Gmelina* sawdust. Fat contents of the mushrooms showed a positive and significant correlation with the cellulose content of the waste. Oil palm fruit fibre yielded the highest quality mushrooms, with 26% in the >7 cm group while GSD and OPEFB had 0% in the same quality group. Considering the desirable characteristics of yield, protein content and market quality, OPFF proved to be a good substrate which could be exploited for large scale production of *L. squarrosulus*.

Key words: *Lentinus squarrosulus*, yield, market quality, crop cycle time.

INTRODUCTION

Huge quantities of a wide variety of organic wastes are generated annually through the activities of the agricultural, forest and processing industries. Sadly, much of these wastes are either burnt, shredded or used as landfill even though these wastes constitute a potentially valuable resource and can be recycled for the production of edible food in the form of mushrooms for man (Chang, 1996). Oil palm fruit fibre and empty bunches are the major components of all solid waste produced from the palm oil industry. These palm oil wastes are heterogeneous water

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Abbreviations: MSD, Mahogany sawdust; **GSD,** Gmelina sawdust; **OPFF,** oil palm fruit fibre; **OPEFB,** oil palm empty fruit bunch; **PDA,** potato dextrose agar; **CRD,** completely randomized design; **DMRT,** Duncan multiple range test.

insoluble materials consisting of cellulose, hemicelluloses and lignin and to a lesser extent pectin, starch and other polysaccharides (Thomsen, 2005). The fruit fibre has been shown to possess high potential to be used as mushroom growing substrate without any further treatment (Abd Razak et al., 2012). On the other hand, sawdust is the most popular basal ingredient used in the formulation of substrate for producing various types of mushroom and is abundantly available during timber processing. Christopher and Custodio (2004) revealed that hardwoods like mahogany contain much higher amounts of structural carbohydrate than softwood trees and hence have more nutrients that could be utilized by mushrooms for their growth and fructification.

Lentinus squarrosulus is a highly prized Nigerian mushroom, which is appreciated for its meaty taste and texture (Kadiri, 2005). The fungus is widely distributed across sub- Saharan Africa and many parts of Asia and is currently attracting interests due to its rapid mycelia growth and potential for use in food and biodegradation (Isikhuemhen et al., 2012). Several reports have shown that various lignocellulosic residues from agro-industrial sector, such as oil palm and timber wastes among others, can provide this mushroom with nutrients required for its spawn run and fructification which under controlled conditions and procedures result in an optimum mushroom yield (Okhuoya et al., 2005; Ayodele and Akpaja, 2007). Nutritional compositions of mushrooms are known to vary with strain, substrates, cultivation and fruiting conditions and the developmental stage of the mushroom (Jo et al., 2013). Due to the development of varying cultivation techniques which in turn affect the chemical compositions of mushrooms, new nutritional data are needed, based on the substrates and the cultivation protocols adopted. The global upsurge in the market for mushrooms could be attributed to their culinary, nutritional and medicinal properties. However, in order to exploit the market potentials of this group of fungi, there is need for better understanding of the influence of locally available organic residues on their marketable qualities such as pilei and stipe sizes. Innovations in cultivation using the abundant low value agricultural residues in Africa could open up new market opportunities through the production of high quality mushrooms with higher demands.

In the present study, four locally available organic wastes; oil palm fruit fibre, oil palm empty fruit bunch, sawdust of mahogany and *Gmelina arborea* were utilized for the cultivation of *L. squarrosulus*. The influence of these wastes on the crop cycle time, yield, nutrient compositions and market quality of the fruit bodies were evaluated.

MATERIALS AND METHODS

Sources of materials

Stock culture of *L. squarrosulus* used for this study was obtained from the Pathology Unit of Forestry Research Institute Ibadan, Nigeria. The culture was maintained on potato dextrose agar (PDA, Difco) until used for spawn preparation. Fresh hardwood sawdust of mahogany (*Khaya ivorensis*) and *G. aborea* were collected from a local sawmill in Nsukka, Nigeria while the oil palm empty fruit bunch (OPEFB) and oil palm fruit fibre (OPFF) were sourced from a local oil palm industry in Nru, Nsukka, Nigeria.

Spawn preparation

The spawn was prepared using Sorghum (*Sorghum bicolor*) grains as substrate. Jars measuring 11 × 9 cm each were filled with parboiled sorghum grains (75% water) to three quarter full and covered with cotton wool. The jars were autoclaved at a temperature of 121°C and 103 kPa pressure for 1 h and allowed to cool down overnight. Each jar was inoculated with the mycelium growing in a 9 cm diameter Petri dish under aseptic conditions on a clean bench. The inoculated grains were incubated at 25 ± 2°C for one to two weeks in the dark. After the grains had been fully colonized, they were stored in a refrigerator at 4°C until required.

Substrate preparation and spawning

Fresh OPEFB chopped into small pieces of about 1 to 5 cm and OPFF were soaked in distilled water overnight in order to wash out the remaining oil in the fibre and to gain 75% moisture content (Chiejina and Olufukunbi, 2010). The moisture content of the sawdust was adjusted to 75% with distilled water by sprinkling using the squeeze test method (Oei, 1991). Three hundred grams oven-dry-weight equivalent of the moistened substrates were filled into 10 high porosity polypropylene plastic bags measuring 17.5 × 15 cm each. Thereafter, the mouth of each bag was plugged with cotton wool and covered with aluminium foil paper. The bags were autoclaved at a temperature of 121°C and 103 kPa pressure for 1.5 h. Sterilized substrates were allowed to cool down to ambient temperature. The bags were randomly picked and spawned with 25 g spawn per 500 g substrate (5%, w/w) under aseptic conditions and incubated at a temperature of 25 ± 2°C in the dark.

Fruit body induction and harvesting

After complete mycelia colonization of the substrates, bags were exposed to temperature of 25 ± 2°C with a 12 h photoperiod in a mushroom growth room. Fruiting bags were sprayed daily with sterile water using a hand sprinkler and free water was placed in a reservoir on the floor to maintain high humidity. Fresh air was circulated in the growing room using an electric fan. Fruit bodies were harvested three days after primordia emergence when the lamellae were fully exposed. Average number of days to spawn run, primordia initiation and fruit body development were recorded. Stipe height and pileus diameter of harvested fruit bodies were measured and expressed in (cm) while yield was the average fresh weight of the mushrooms harvested per kilogram dry weight of substrate and expressed in (g/kg substrate). The market quality of the harvested basidiocarps was evaluated as described by Rossi et al. (2003). Marketable mushrooms were more than 3 cm in diameter and those of highest commercial value were those with diameter of more than 5 cm.

Analytical methods

Cellulose, hemicellulose, lignin (Datta, 1981) and nitrogen (N) (AOAC, 2002) contents of the organic waste substrates were determined before mushroom cultivation on an air dry weight basis. Moisture content of mushrooms was determined by direct

Table 1. Chemical analysis of the main constituents of oil palm fruit fibre (OPFF), oil palm empty fruit bunch (OPEFB), *Gmelina* sawdust (GSD) and mahogany sawdust (MSD) before mushroom cultivation.

Substrate	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Nitrogen (%)
OPFF	44.29	12.00	8.50	1.36
OPEFB	42.86	11.50	10.00	0.69
GSD	27.14	13.50	17.50	0.30
MSD	51.71	13.00	19.30	0.20

Table 2. Mean crop cycle time of *Lentinus squarrosulus* mushrooms grown on oil palm fruit fibre (OPFF), oil palm empty fruit bunch (OPEFB), *Gmelina* sawdust (GSD) and mahogany sawdust (MSD).

Substrate	Spawn run (days)	Primordia induction period (days)	Fruit body formation (days)
OPFF	6.40^{d}	39.40^{a}	48.80^{a}
OPEFB	9.90^{c}	34.40^{b}	47.30^{ab}
GSD	15.40^{a}	28.20^{c}	46.60^{b}
MSD	12.30^{b}	33.30^{b}	48.60^{a}

* Means followed by the same letter(s) in the same column are not significantly different at $p > 0.05$ according to DMRT.

oven-drying to constant weight at 105°C in a hot air oven. Mushroom samples harvested from the same substrates were mixed and homogenised before analyses. The fruiting bodies were oven dried at 60°C in a hot air oven and analysed for nutrient contents; total nitrogen, crude fibre, ash and crude fat using standard procedures (AOAC, 2002). The crude protein content was calculated by using the adjusted conversion factor (N x 4.38) for mushroom protein, due to the significant content of non-protein nitrogen in the form of glucosamine in mushrooms chitinous cell walls (Shashirekha et al., 2005).

Experimental design and data analysis

The experimental design was a completely randomized design (CRD) with 10 replicates. Data were subjected to one way analysis of variance (ANOVA) using SPSS program. Mean separation was done using Duncan multiple range test (DMRT). All analysis was done at 5% level of significance. Correlation analyses were carried out to ascertain the level of relationship between the constituents of the organic waste substrates and mushroom growth, yield and nutrient compositions.

RESULTS AND DISCUSSION

Chemical analyses revealed that initial substrate composition varied among the organic wastes substrates evaluated in this study (Table 1). Total nitrogen content (N) determined in OPFF was found to be higher than values recorded for the other wastes. Cellulose contents of the wastes substrates varied between 27.14 and 51.71% in GSD and MSD, respectively. Tshinyangu and Hennebert (1995) demonstrated that the nutrient composition of substrates is one of the most important factors limiting saprobiotic colonization and fruiting of cultivated mushrooms. The influence of the different organic waste substrates on the duration of colonization phase and primordia induction period of the mushroom is presented on Table 2. OPFF supported the fastest substrate colonization for the mushroom followed by OPEFB, while spawn run was completed after 13 and 16 days on MSD and GSD, respectively. The shortest spawn run on OPFF could be attributed to the waste providing adequate aeration for the growth of the mushroom mycelia. Tinoco et al. (2001) observed that the larger the surface area and pore size of substrates, the more the mycelial growth rate. In the present study, spawn run showed significant positive correlation with lignin content of the waste (Table 5). It is however reasonable to assume that the low lignin content of OPFF could have resulted to the vigorous colonization of the waste by the fungus. Previous studies had shown that high lignin contents of substrates impede mycelial growth as cellulose may not be readily available as carbon source (Freer and Detroy, 1982).

From results presented in Table 2, GSD supported the shortest primordia induction period (29 days) despite presenting a long colonization phase which lasted for 16 days, while on OPFF the pin initiation process commenced considerably later (after 40 days). Results of the correlation studies show that primordia induction period was positively correlated with N content of the substrates (Table 5). It is therefore rational to assume that the N content of GSD may have played a significant role in the earliness of primordia induction of the mushroom on the waste. This is also the case of OPFF where the high N content of the waste had a negative effect on the primordia induction period despite emerging as the best substrate for spawn run. This result therefore confirms

Table 3. Mean stipe height (cm), pilei diameter (cm), yield (g/kg substrate) and dry weights of *L. squarrosulus* harvested from mahogany sawdust (MSD), *Gmelina* sawdust (GSD), oil palm fruit fibre (OPFF) and oil palm empty fruit bunch (OPEFB).

Substrate	Stipe height (cm)	Pileus diameter (cm)	Yield (g/kg substrate)	Dry weight (g)
MSD	4.50[a]	4.86[b]	16.05[b]	2.78[b]
GSD	2.46[c]	4.06[c]	9.02[c]	1.56[c]
OPFF	3.17[b]	5.76[a]	30.10[a]	4.31[a]
OPEFB	2.10[c]	3.01[d]	4.12[d]	1.06[c]

*Each value is a mean of 10 replicates. Values in the same column followed by the same letter (s) are not significantly different at p > 0.05 according to DMRT.

Table 4. Nutrient compositions of harvested mushrooms from Oil palm fruit fibre (OPFF), Oil palm empty fruit bunch (OPEFB), *Gmelina* sawdust (GSD) and Mahogany sawdust (GSD).

Organic wastes	Protein content (% d.w.)	Crude fibre (% d.w.)	Ash (% d.w.)	Moisture (% d.w.)	Fat (% d.w.)
OPFF	27.42[a]	5.09[a,b]	7.34[a,b]	12.73[b]	0.68[b]
OPEFB	17.57[b]	4.58[b]	6.86[b]	13.33[a]	0.39[c]
GSD	19.79[b]	5.40[a]	7.97[a]	12.28[b]	0.38[c]
MSD	13.27[c]	5.17[a]	6.71[b]	13.33[a]	0.91[a]

*Values (means of at least three replicates) in the same column followed by the same letter (s) are not significantly different at p > 0.05 according to DMRT.

Table 5. Simple correlations between constituents of the organic waste substrates and mushroom growth, yield and nutrient compositions.

Properties	Nitrogen (%)	Cellulose (%)	Lignin (%)	Hemicellulose (%)
Spawn run	-0.9053**	-0.5449	0.8485**	0.8110**
Pin initiation	0.8532**	0.6444*	-0.7491*	-0.7352*
Yield	0.6672*	0.3539	-0.3023	-0.0687
Protein	0.8821**	-0.2630	-0.7108*	-0.2992
Fat	-0.0363	0.7934**	0.3086	0.1505
Fibre	-0.3048	-0.4038	0.6332*	0.9078**
Moisture	-0.0982	0.8473**	-0.0807	-0.5364
Ash	0.0422	-0.9171**	0.0768	0.5114
Pilei diameter	0.4458	0.3167	-0.0348	0.1873

*Significant at P < 0.05, **Significant at P < 0.01

the conclusion previously drawn by Zadrazil and Brunnert (1979) that although N increases yields, above a certain level, it inhibits fruiting. The highest yield (30.10 g/kg substrate) in the current study was obtained from OPFF (Table 3). MSD and GSD also furnished significantly higher crop yields than OPEFB, which was also the least productive as regards mushroom height and pilei diameter. Fruit bodies with the widest pilei diameter and highest stipe height were recorded on OPFF and MSD respectively (Table 3). Correlation studies revealed that yield was positively correlated with N contents of the waste substrates (Table 5). Although, the higher N content of OPFF may have contributed to the significant yield of the mushroom, probably, the residual oil from the waste could also have played an important role in promoting higher yield of the mushroom on OPFF. Earlier reports had shown that plant oils are stimulatory to mushroom mycelial growth and sporophore production, since they are needed for expanding cell membranes and hence result in higher yields (Schisler, 1967; Song et al., 1989). The lowest yield recorded for fruit bodies harvested from OPEFB could be attributed to the complex nature of the waste and/or the presence or little or no vital nutrients needed for mushroom growth in the substrate.

Table 4 shows the influence of the organic waste substrates on the nutritional content of *L. squarrosulus*. Chemical composition of the substrates used in cultivation

Table 6. Substrate effect of *Gmelina* sawdust (GSD), mahogany sawdust (MSD), oil palm empty fruit bunch (OPEFB) and oil palm fruit fibre (OPFF) on *Lentinus squarrosulus* market quality evaluated by cap size group.

Substrate	Market quality groups (cm)				
	<3 cm	3-5 cm	5-7 cm	>7 cm	Deformed
GSD	8.00^{b2}	82.00^{a1}	10.00^{b2}	0.00^{b2}	0.00^{a2}
OPFF	14.00^{b2}	36.00^{c1}	34.00^{a1}	26.00^{a1}	0.00^{a3}
OPEFB	44.00^{a2}	56.00^{b1}	0.00^{b3}	0.00^{b3}	0.00^{a3}
MSD	8.00^{b3}	52.00^{b1}	30.00^{a2}	10.00^{b3}	0.00^{a3}

*Values are expressed as a percentage of the pileus diameter and represent the mean of 10 replicates. Mean values in the same row followed by the same figure (s) are not significantly different at $p > 0.05$. Mean values in the same column followed by the same alphabet (s) are not significantly different at $p > 0.05$ according to DMRT.

has been shown to have a direct effect on the chemical composition of the fruit bodies (Shashirekha et al., 2005). In the present study, protein, crude fibre, ash, moisture and fat contents of the fruit bodies were affected by the substrates. OPFF produced mushrooms with the highest protein content (27.42%) followed by GSD (19.79%) while MSD yielded mushrooms with the lowest protein (13.27%). The fruit bodies of *L*. *squarrosulus* grown on GSD were richer in crude fibre and ash than those harvested from the other waste substrates evaluated in this study. The variations in the protein content of fruit bodies harvested from the different substrates may be attributed to the differential availability of usable nitrogen after spawn run, which in turn influenced the amount of nitrogen available for utilization during sporophore development. From the results presented in Table 5, it is evident that protein content of fruit bodies was positively correlated with N contents of the substrates. This positive relationship justifies the importance of N contents of mushroom cultivation substrates in the production of protein rich fruit bodies. Moreover, N has been reported to be an important nutrient required for fungal growth due to its involvement in protein, chitin and nucleic acid synthesis (Adebayo et al., 2009). In the present study, mushrooms with the lowest fat content (0.38%) were harvested from GSD while MSD stimulated the production of fruit bodies with the highest fat content (0.91%). One of the main reasons why mushroom is a favoured item for human nutrition is its abundance of unsaturated fatty acids and its hypocholestrolemic property (Shashirekha et al., 2005).

Correlation studies between the constituents of the different organic waste substrates and the growth parameters of the mushroom (Table 5) revealed a positive correlation of spawn run with hemicellulose content. Primordia induction period was also found to be positively correlated to cellulose content. These findings were further supported by Moyson and Verachtert (1991) who demonstrated that substrate decomposition by *Lentinula edodes* is initially associated with its hemicellulose content. Gaitan-Hernandez et al. (2011) also reported a positive correlation of days to primordia formation with cellulose content indicating that cellulose in each substrate was directly proportional to primordia formation. In the current study, fibre content of the fruit bodies were significantly positively related with hemicellulose content of the wastes. Fat and moisture contents of the mushroom were also found to be positively correlated with cellulose. However, spawn run showed a significant negative correlation with N content of the substrates. Primordia induction period was also negatively correlated with lignin and hemicellulose contents of the waste. Mushroom market quality in terms of basidiocarp pileus diameter was affected by substrate type (Table 6). OPFF yielded the highest quality mushrooms, with 26% in the >7 cm group while GSD and OPEFB had none in the same quality group (Table 6). GSD stimulated highest production (82%) of basidiocarp in the 3 to 5 cm size group (Table 6). OPEFB had many of the basidiocarps in the 3 to 5 and <3 cm size groups with 56 and 44%, respectively (Table 6). It is noteworthy that no fruit bodies were deformed throughout the experiment and hence none was recorded for the deformed group. Mushroom size is essential for its market evaluation since mushrooms with wide pilei could be of interest in the promotion of mushroom marketing. Although, the large sized fruit bodies harvested from OPFF and MSD are considered to be of good market quality and are rated highly, Shen and Royse (2001) observed that this could be an inferior quality since such fruit bodies tend to break during packaging thereby reducing their quality. In the present study, GSD and OPEFB also produced much of the mushrooms in the 3 to 5 cm quality group which are also marketable. The differences in mushroom quality in the different waste substrates could be due to the nutrient status and the nature of lignocellulose in the respective wastes. This observation corroborates the findings of Fung et al. (2005) who demonstrated that nutrient

concentration in the substrates determined the productivity and quality of the mushroom crop.

Conclusion

The results of the present study indicate that unsupplemented and uncomposted locally available organic wastes (MSD, GSD, OPFF and OPEFB) could support the growth and fructification of *L. squarrosulus*. It also showed that *L. squarrosulus* with good yields, high protein and market quality could be cultivated using OPFF. This is of particular interest in the developing countries where the availability of cheap sources of protein is paramount in order to counter protein malnutrition. The current study also shows that the overall nutritional properties of the fruit bodies harvested from GSD is promising. However, due to the low yield recorded on GSD, there is need for detailed investigations on the supplementation of this waste with nitrogen sources to increase yield.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

Abd Razak DL, Abdullah N, Khir Johari NM, Sabaratnam V (2012). Comparative study of mycelia growth and sporophore yield of *Auricularia polytricha* (Mont.) Sacc on selected palm oil wastes as fruiting substrate. Appl. Microbiol. Biotechnol. 97:3207-3213.

Adebayo GJ, Omolara BN, Toyin AE (2009). Evaluation of yield of oyster mushroom (*Pleurotus pulmonarius*) grown on cotton waste and cassava peel. Afr. J. Biotechnol. 8(2):215-218.

AOAC (2002). Official Methods of Analysis (17th Edition). Association of Official Analytical Chemist, Maryland, 1106pp.

Ayodele SM, Akpaja EO (2007). Yield evaluation of *Lentinus squarrosulus* (Mont) Sing. on selected sawdust of economic tree species supplemented with 20% oil palm fruit fibers. Asian J. Plant Sci. 6 (7):1098-1102.

Chiejina NV, Olufokunbi JO (2010). Effects of different substrates on the yield and protein content of *Pleurotus tuberregium*. Afr. J. Biotechnol. 9 (11):1573-1577.

Chang ST (1996). Mushroom biology: the impact on mushroom production and mushroom products. In: Royse, J. (Ed.). Proceedings of the 2nd International Conference on Mushroom Biology and Mushroom Products. University Park, Pennsylvania, USA. pp. 3-20.

Christopher J, Custodio D (2004). Coco lumber sawdust. In: Gush R (Ed.). Mushroom Grower's Handbook I. Mushworld Inc.

Datta R (1981). Acidogenic fermentation of lignocellulose-acid yield and conversion of components. Biotechnol. Bioeng. 23(9):2167-2170.

Freer SN, Detroy RW (1982). Biological delignification of ^{14}C-labeled lignocelluloses by basidiomycetes: degradation and solubilization of the lignin and cellulose components. Mycologia 74(6):943-951.

Fung YW, Fung TW, Franco M (2005). Evaluation of different Colombian agroindustrial wastes as substrates for the growth and production of *Lentinula edodes* Berk. Pegler (shiitake). Acta Edulis Fungi 12:285-290.

Gaitan-Hernandez R, Esqueda M, Gutierrez A, Beltran-Garcia M (2011). Quantitative changes in the biochemical composition of lignocellulosic residues during the vegetative growth of *Lentinula edodes*. Braz. J. Microbiol. 42:30-40.

Isikhuemhen OS, Mikiashvili NA, Adenipekun CO, Ohimain EI, Shahbazi G (2012). The tropical white rot fungus, *Lentinus squarrosulus* Mont. lignocellulolytic enzymes activities and sugar release from cornstalks under solid state fermentation. World J. Microbiol. Biotechnol. 28:1961-1966.

Jo EY, Choi JY, Choi JW, Ahn JH (2013). Influence of food waste compost on the yield and mineral content of *Ganoderma lucidium*, *Lentinula edodes*, and *Pholiota adipose* fruiting bodies. Mycobiology 41(4):210-213.

Kadiri M (2005). Toxicological evaluation of *Lentinus squarrosulus* Mont. (Polyporales), an indigenous Nigerian mushroom. Int. J. Med. Mushrooms 7(3):416-417.

Moyson E, Verachtert H (1991). Growth of higher fungi on wheat straw and their impact on the digestibility of the substrate. Appl. Microbiol. Biotechnol. 36:421-424.

Oei P (1991). Manual on Mushroom Cultivation: Techniques, Species and Opportunities for Commercial Application in Developing Countries. Tool Publications, Netherlands. 329pp.

Okhuoya JA, Akpaja EO, Oghenekaro A (2005). Cultivation of *Lentinus squarrosulus* (Mont.) Singer on sawdust of selected tropical tree species. Int. J. Med. Mushrooms 7(3):440-441.

Rossi IH, Monteiro AC, Machado JO, Andrioli JL, Barbosa JC (2003). Shiitake (*Lentinula edodes*) production on a sterilized baggase substrate enriched with rice bran and sugarcane molasses. Braz. J. Microbiol. 34:66-71.

Schisler LC (1967). Stimulation of yield in the cultivated mushroom by vegetable oils. Appl. Microbiol. 15(4):844-850.

Shashirekha MN, Rajarathnam S, Bano Z (2005). Effects of supplementing rice straw growth substrate with cotton seeds on the analytical characteristics of the mushroom, *Pleurotus florida* (Block & Tsao). Food Chem. 92:255-259.

Shen Q, Royse DJ (2001). Effects of nutrient supplements on biological efficiency, quality and crop cycle time of maitake (*Grifola frondosa*). Appl. Microbiol. Biotechnol. 57(1-2):74-78.

Song CH, Cho KY, Nair NG, Vine J (1989). Growth stimulation and lipid synthesis in *Lentinus edodes*. Mycologia 81(4):514-522.

Thomsen MH (2005). Complex media from processing of agricultural crops for microbial fermentation. Appl. Microbiol. Biotechnol. 68:598-606.

Tinoco R, Pickard MA, Vazquez-Duhalt R (2001). Kinetic differences of purified laccases from six *Pleurotus ostreatus* strains. Lett. Appl. Microbiol. 32(5):331-335.

Tshinyangu KK, Hennebert GL (1995). Effect of synthetic nutrient carriers on the fruiting of *Pleurotus ostreatus* var. *columbinus*. Bioresour. Technol. 54(3):249-254.

Zadrazil F, Brunnert H (1979). Influence of ammonium nitrate on the growth and straw decomposition of higher fungi. Zeit Pflanzenernaehr Bodenkd 142:446-455.

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Catalase activity of cassava (*Manihot esculenta*) plant under African cassava mosaic virus infection in Cape coast, Ghana

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African cassava mosaic virus has caused an immersed low yield of the cassava crop. The virus impacts stress on the cellular metabolism of the plant producing a lot of reactive oxygen species and increases the expression of the antioxidant enzymes. The activity of catalase as a response to oxidative stress was investigated in this research. Cassava leaf extract infected with the African cassava mosaic virus and uninfected were prepared and used as crude catalase extract. The total protein was determined by Biuret method and activity of the crude catalase was compared using hydrogen peroxide as substrate. Infected leaf extract had 141.02 ± 3.536 mg/mL protein, apparent Michealis constant (Kmapp) of 26.7 µM and maximum rate of reaction (Vmax) of 54.50 µM/min compared to the uninfected leaf extract with 75.04 ± 0.560 mg/mL protein, Kmapp of 39.61 µM and Vmax of 143.06 µM/min. The activation energy of the infected extract was 0.1578 J/mol compared to 0.2181 J/mol obtained for the uninfected extract. Activity of the crude catalase in the viral infected leaf extract from the study was higher than that in the uninfected one and confirms the response to the stressful condition imposed by the viral infection.

Key words: Cassava leaf extract, African cassava mosaic virus (ACMV), reactive oxygen species (ROS), catalase, hydrogen peroxide, Kmapp, Vmax.

INTRODUCTION

Cassava (*Manihot esculenta*) is a woody shrub of the Euphorbiaceae that can grow for more than two years. Although, cassava has some features that allow it to cope with stress better than other crops, such as high stomatal sensitivity to environmental humidity, deep rooting capacities and quick recovery after stress under these conditions, productivity is sub-optimal and unstable (El-Sharkawy, 2012). Cassava has therefore become African

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Abbreviations: CBSD, Cassava brown streak disease; **ACMV,** African cassava mosaic virus; **ROS,** reactive oxygen species; **H_2O_2,** hydrogen peroxide; **BSA,** bovine serum albumin.

continent's most important food security crop and a major source of carbohydrates. In Ghana, cultivation of cassava is a reserve crop against lean periods, because it can survive all weather conditions and it is the last resort of food, when there is severe shortage of other food crops. Several items of foods are processed from cassava, examples are: fufu, gari, cassava dough, starch, bread, biscuits and cassava flour (konkonte).

Cassava productivity is threatened by abiotic factors such as temperature, soil conditions, pH and rainfall and also biotic factors such as bacterial and viral diseases, as well as arthropod pests. The most important diseases include cassava bacterial blight, *Xanthomonas campestris manihoti*, African cassava mosaic disease and cassava brown streak disease (CBSD), the two viral diseases (Parkes et al., 2013) both of which their infection are thought to have risen from viruses already present in the crop through propagation (Njock, 2014). In Africa, this is extremely detrimental to the production of subsistence farming. Cassava plants infected with the African cassava mosaic virus (ACMV) express a range of symptoms which depend on the virus strain, environmental conditions, and the sensitivity of the cassava host. African cassava mosaic virus (ACMV) is a single strand DNA pathogenic virus transmitted by the whitefly *Bemisia tabaci*. ACMV causes either a mosaic appearance to plant leaves or chlorosis and a loss of chlorophyll. In *M. esculenta* (cassava), a highly valuable African food crop, the virus is the only known plant virus that causes cassava mosaic disease (CMD). When plant is under a stress condition, like a viral infection, there is increased rate of photosynthesis and respiration. These results in the release of reactive oxygen species (ROS) mainly hydrogen peroxide (H_2O_2) which causes membrane damage that eventually leads to cell death which is fatal to plant life. When there is an imbalance (either by abiotic or biotic factors) in the cellular compartment between ROS production and antioxidant defense, there is dramatic physiological challenges which results in oxidative stress. The generation of H_2O_2 seems to be mediated by a membrane-bound NADPH oxidase complex in plants. H_2O_2 may further activate defense genes such as proteinase inhibitors as it diffuses to adjacent cells. Environmental stresses that cause oxidative stress include drought, salt stress, extreme temperatures, air pollution, oxidant-forming herbicides, heavy metals, wounding, UV light, and high intensity light conditions that stimulate photo-inhibition of photosynthesis.

During oxidative stress, the plant protects itself against reactive oxygen species by antioxidant enzymes as well as a wide array of non-enzymatic antioxidants (Das and Roychoudhury, 2014). Superoxide dismutase is considered to be the first line of defense against ROS and is the major O_2^- scavenger. Its enzymatic action results in H_2O_2 and O_2 formation. The H_2O_2 produced is then scavenged by catalase and several classes of peroxidases. Peroxidases decompose H_2O_2 by the oxidation of phenolic compounds. The catalase is a tetrameric heme protein, found in peroxisomes, cytosol and mitochondria (Krych et al., 2014). This enzyme has hyperoxidase activity which catalyzes the dismutation of hydrogen peroxide into water and oxygen. Catalase is nearly ubiquitous among organisms that can grow in the presence of oxygen. The major function of catalase within cells is to prevent the accumulation of toxic levels of hydrogen peroxide formed as a by-product of metabolic processes - primarily that of the electron transport pathway.

The objectives of the study was to compare protein levels in virus infected and uninfected leaf extracts of cassava plants and also to determine the kinetic parameters: Kmapp and Vmax and the activation energy of crude catalase extract from the different portions of the cassava leaf under study.

MATERIALS AND METHODS

Collection and handling of cassava leaves

Leaves of the infected cassava ("Obotan": local name for the type of cassava used) showing the symptoms of mosaic virus and the non-mosaic patterns were collected from the University of Cape Coast, Department of Agriculture School Farm. The plants were specific for CMD from the Agricultural Department and free of co-infection. The two leaf samples were collected from different positions of the branches on the same plants with the disease. They were transported to the laboratory in labeled rubber bags where they were washed with water and stored in the refrigerator prior to use.

Homogenization of cassava leaves

The vein of the leaf was cut out and 3.0 g of each leaf sample weighed on a balance. With a chilled mortar and pestle, the weighed leaves were macerated in 20 mL each of 0.1 M universal buffer containing 0.1 M citric acid·H_2O, 0.1 M potassium phosphate monobasic, 0.1 M sodium tetraborate·10H_2O, 0.1 M Tris and 0.1 M potassium chloride adjusted to pH 7. The homogenate was then centrifuged at 12000 rpm for 30 min at 0°C. The supernatant was decanted and aliquoted into Eppendoff tubes. The aliquots were stored frozen prior to use as the crude enzyme extract.

Determination of protein in the crude enzyme extract of the leaf

The concentration of protein in the crude extract was determined from an absorbance-concentration calibration graph using standard bovine serum albumin (BSA) solutions prepared from a stock solution of 15 mg/mL.

The kinetics of crude catalase activity

Preliminary investigations were made to determine the appropriate reaction times as well as the amount of the crude enzyme extract to be used for the kinetic studies. From the investigations, a reaction time of 5 min and crude enzyme extract of 0.3 mL were used for the subsequent investigations. A set of eight clean test tubes were obtained and into each tube, 1.7 mL of the universal buffer at pH of 7 and 0.3 mL of the crude enzyme extract was added. A water-bath was set and tubes were incubated at 90°C for 5 min to denature the enzyme present. The test tubes were then removed after the 5 min

Table 1. Crude protein determination in leaf extracts.

Leaf extract	Protein concentration ($mgmL^{-1}$)
Obotan with virus	141.02 ± 3.536
Obotan without virus	75.04 ± 0.560

Table 2. Change in absorbance of H_2O_2 for extracts.

H_2O_2 (mg/mL)	Absorbance	
	ΔAb H_2O_2 Used for reaction (uninfected virus extract)	**Δ Ab H_2O_2 Used for reaction (virus infected extract)**
0.04	0.0679	0.0714
0.08	0.1298	0.1324
0.12	0.1681	0.1707
0.16	0.2785	0.2789
0.20	0.3061	0.3053
0.24	0.3446	0.3428
0.28	0.4126	0.4107
0.32	0.4853	0.4832

and allowed to cool to room temperature. Serial dilutions of the 0.3% H_2O_2 were done. 2 mL of each dilution was measured into labelled test tubes and the absorbance read at a wavelength of 240 nm. Into another set of eight test tubes were added, 2 mL of the H_2O_2 serial dilutions and 1.7 mL of the buffer. 0.3 mL of the thawed crude extract (frozen activated extract) was added into each tube at time interval of 5 min at room temperature. At the 5^{th} minute, the absorbance of each reaction was recorded at 240 nm. The absorbance of the second set of test tubes (activated; enzymes present) was subtracted from the first set of test tubes (inactivated; enzymes denatured) to remove the effects of the color of the extract due to H_2O_2. The final absorbance obtained was then subtracted from that of the initial H_2O_2 concentration to obtain the change in absorbance.

Kmapp and Vmax of crude catalase activity of leaf extracts

The change in absorbance obtained was converted to concentration (µM) using Beer-Lambert's law, A=ƐCl. The concentration obtained was divided by the reaction time (5 min) to obtain the rate of the reaction. Specific activity was also determined by dividing the rate by the total protein concentration of each crude enzyme extract. The reciprocal of both the substrate concentration and the rate of activity were determined and Lineweaver-Burk plot was plotted from which the Kmapp and Vmax were determined.

Activation energy of the crude catalase activity in the leaf extracts

The effect of temperature on the activity of the crude catalase extracts were investigated within a temperature range of 30 to 60°C. A serial dilution (0.2 mg/mL) of the stock solution of 0.3% H_2O_2 was prepared and 2 mL was added to 1.7 mL of the universal buffer at pH of 7 and placed in a set, water bath at 30°C. When the mixture has attained the temperature of the water bath (30°C), 0.3 mL of the crude extract was added and allowed to react for 5 min after which the absorbance was read at 240 nm. The procedure above was repeated for a denatured enzyme extract (heated at 90°C for 5 min). The absorbance of the first test tube (activated) was subtracted from the second test tube (inactivated) to remove the effect of the color of the extract on the reaction. The procedure as described above was also carried at temperatures 40, 50 and 60°C, respectively.

The rate and specific activity of the enzymatic activity was determined by converting the absorbance to molar concentration using Beer-Lambert's law and the log of the rate found. The Arrhenius relation was plotted from which the activation energy of the crude catalase extract was extrapolated.

RESULTS

Protein determination in crude leaf extracts

To determine the protein content in the Obotan leaf extracts, the absorbance readings were plotted and the concentration of the leaf extracts were extrapolated from the standard BSA absorbance plot. Values of concentration with their means were determined (±the standard deviation) as shown in Table 1.

Crude catalase activity in leaf extracts

The absorbance of H_2O_2 left after reaction was subtracted from initial absorbance to attain the change in absorbance of H_2O_2 used for the reaction and the result shown below for the crude extracts (Table 2).

Kinetics of crude catalase activity

The absorbance was converted to molar concentration using the Beer Lambert's Law (A=ƐCl). The rate, specific

Table 3. Kmapp and Vmax for crude catalase activity.

Leaf extract	Kmapp (µM)	Vmax (µM/min)
Obotan with ACMV infection	26.70	54.50
Obotan without viral infection	39.61	143.06

Table 4. Arrhenius data of crude catalase extracts.

Temperature	Leaves extract with virus infection	Leaves extract without infection
$1/Tx10^{-3}\ K^{-1}$	Log R	Log R
3.299	0.771	1.217
3.193	0.719	1.221
3.095	0.742	1.262
3.002	0.749	1.302

Table 5. Activation energy of crude catalase activity in leaf extracts.

Leaf extract	Activation energy (J/mol)
Obotan with ACMV infection	0.1578
Obotan without viral infection	0.2181

activity and the inverse of the rate were determined for each leaf extract. A double reciprocal (Lineweaver-Burk's) was plotted and the kinetic parameters determined from the plot. The line of equation for Obotan with virus and without virus were y = 4.899x + 0.1835 and y = 2.7689x + 0.0699, respectively. These equations were compared with the equation of line for Lineweaver-Burk plot. The Kmapp and Vmax for both extracts were then determined as shown in Table 3. The change in absorbance used for the reaction was converted to molar concentration using the Beer Lambert's Law (A=ƐCl). The rate, specific activity and the logarithm of the rate were determined for each leaf extract. The temperature in degree Celsius was also converted to degree Kelvin and the inverse determined. Arrhenius plot was done and the activation energy determined from the slope by comparing the line of equation with log (Tables 4 and 5).

R = - Ea(1/T) + Yo.

DISCUSSION

Plants under stressful conditions tend to respond in order to survive these conditions homeostatically. Hydrogen peroxide is a primary metabolites produced excessively in green plants when under stress. At lower concentrations, they tend to protect the plant from pest. Therefore, to investigate the activity of catalase, the protein in the two leaf extract was determined by the biuret method and the results obtained were compared. The protein concentration determined were 141.02 ± 3.536 mg/mL in the extract of the viral infected leaves and 75.04 ± 0.560 mg/mL in the healthy leaves. Studies by Coldebella et al. (2013) showed that cassava healthy leaves are rich in protein but difference in the protein concentration as observed in this study was because of increased viral protein on the infected leaves as compared to the uninfected one. Again the plant under study was infected by the virus hence lowering its protein content in leaves. The kinetic parameters of the crude enzyme extract from the viral infected and the healthy leaves were also studied. The solutions of the hydrogen peroxide reacted with the extract from the viral infected leaves changed from yellow to straw-like color after the reaction time (Figure 1) whilst that with the extract from the healthy leaves remained yellow. The change in color as observed was as a result of activity of the reactive oxygen species (ROS- H_2O_2 and other free radicals) on the plant's pigment which could be investigated further. This might be due to a unique reaction which was not present in the non-infected extract since heat treatment was able to stabilize the color change. Hence, another preparation of the hydrogen peroxide and the crude extract from the viral infected leaves were made where the enzyme extract was heated at 90°C to denature the enzyme before adding to the hydrogen peroxide solution. The color change was still observed showing it was not as a result of enzymatic activity. The absorbance of the activity of the crude extract was then deducted from the denatured extract activity in order to obtain the activity on the hydrogen peroxide although it was not that efficient in completely removing the color effect.

From the results obtained, the Line-Weaver Burk plot that was made for each crude enzyme extract had a positive slope from which the Kmapp and the Vmax were determined. Also, the Kmapp for the viral infected leaf extract and the healthy leaf extracts were 26.7 and 39.61 µM,

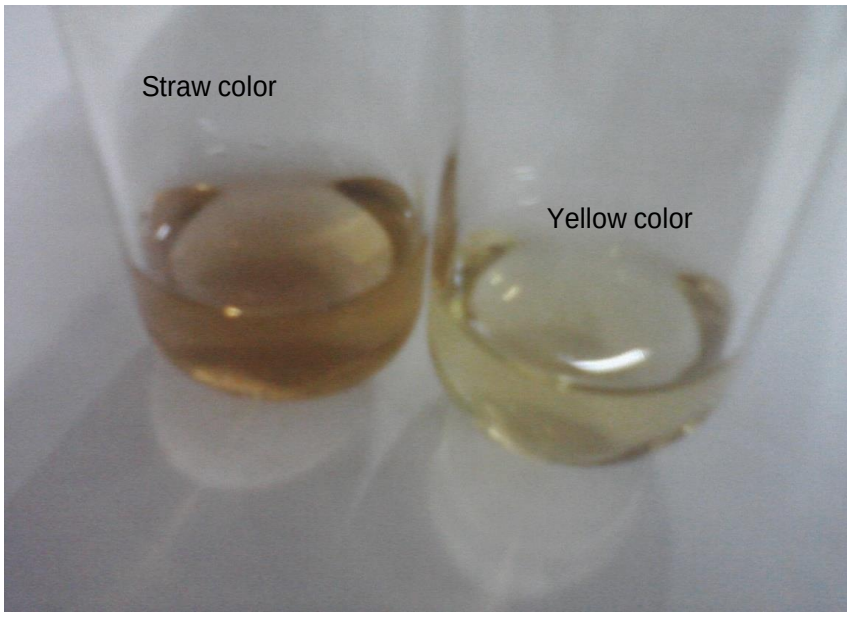

Figure 1. Effect of hydrogen peroxide on the cassava leaf pigment.

respectively. The relative difference in activity measured showed a higher affinity for the hydrogen peroxide in the viral infected leaf compared to the healthy leaf extracts. This observation explains why there is increased expression of catalase in the stressed plant and this is very necessary to rapidly hydrolyze the elevated levels of the hydrogen peroxide so as to prevent any harmful effect and also to promote food security of cassava among the people of Cape Coast, Ghana since it is their most staple food. On the other hand, the Vmax of 143.06 µM/min as against 54.50 µM/min was observed without viral leaf extract and viral leaf extract, respectively. A higher Vmax shows a faster activity and the one with the lower Kmapp was expected to have a higher Vmax as a result of the higher affinity. The deviation could have resulted due to the superimposing color of the reaction mixture.

The activation energy of 0.1578 J/mol was determined for the extract with the viral infection as compared to 0.2181 J/mol for the healthy leaf extracts. The result shows a higher activity at higher temperatures for the viral infected extract. The viral infected extract will be less sensitive to temperature than the viral uninfected extract. Since, the Arrhenius plot was a plot of log of rate against 1/T (K^{-1}), the peroxide concentration did not have a direct influence on the activation energy as it did on the Vmax in the Lineweaver-Burk plot.

Conclusion

From the results and the observations made from the study, it has been shown that there is a significant increase in the protein concentration in the ACMV infected cassava leaves as compared to that in the uninfected one. It was also observed that a lot of hydrogen peroxide were produced in the stressed leaf and hence the increased expression of the catalase activity. This was evident by the lower Kmapp and the lower activation energy which were measured for the ACMV infected cassava leaf extract. It can therefore be concluded that there is an increased level of catalase expression in the cassava leaf infected with the Africa cassava mosaic virus (ACMV) relative to the uninfected one. The study investigators recommend that the plant DNA should be extracted, purified and quantified to ascertain the inducement of the catalase activity. Also, further other antioxidant enzymes such as superoxide dismutase and peroxidase should also be determined to investigate the infected plants. Lastly, the effect of the hydrogen peroxide on the plant pigment after extraction in the viral infected plants should be investigated.

Conflict of interests

The authors did not declare any conflict of interest.

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REFERENCES

Coldebella PF, Gomes SD, Evarini JA, Cereda MP, Coelho SRM, Coldebella A (2013). Evaluation of protein extraction methods to obtain protein concentrate from cassava leaf. Eng. Agric. 33:6.

Das K, Roychoudhury A (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Front. Environ. Sci. 2:53

El-Sharkawy MA (2012). Stress-Tolerant Cassava: The Role of Integrative Ecophysiology-Breeding Research in Crop Improvement. Open J. Soil Sci. 2:162-186.

Krych J, Gebicki JL, Gebicka L (2014). Flavonoid-induced conversion of catalase to its inactive form — Compound II. Free Radic. Res. 48:1334-1341

Njock TE (2014). Evaluation of the infection of cassava and Nicotiana benthamiana with cassava mosaic geminiviruses and their combinations for potential synergism. Global Adv. Res. J. Agric. Sci 3 (10):310-316.

Parkes EY, Fregene M, Dixon A, Boakye-Peprah B, Labuschagne MT (2013). Combining ability of cassava genotypes for cassava mosaic disease and cassava bacterial blight, yield and its related components in two ecological zones in Ghana. Euphytica 194:13-24.

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Effect of collection time on the viability of banana pollen grains

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The objective of this study was to assess the pollen viability of six improved diploid banana plants (AA) collected in different periods of the day, in two seasons of the year (winter and summer), using *in vitro* germination test and staining. Pollen grains collected at 8 am (anthesis), 10 am, 12 pm, 2 pm and 4 pm were evaluated. We used a culture medium for pollen germination containing 15% sucrose, 0.01% H_3BO_3, 0.01% KNO_3, 0.03% $Ca(NO_3)_2.4H_2O$, and 0.02% $MgSO_4.7H_2O$, solidified with 0.8% agar, adjusted to different pH. The pollen viability was evaluated by staining with 2,3,5-triphenyltetrazolium chloride (TTC). The highest pollen germination rates and viability were obtained at 8 am and the lowest at 4 pm, in both seasons. The average *in vitro* germination percentage and viability level were negatively influenced by the number of hours after anthesis. During the summer, the pollen viability and *in vitro* germination rates were highest when compared to winter period. The results presented can help at the selection of genetic materials and enable inferences on the best pollen collection time for use in cross breeding programs for plant improvement.

Key words: *Musa* spp., *in vitro* pollen germination, 2,3,5-triphenyltetrazolium chloride.

INTRODUCTION

In Brazil, the conventional banana breeding program is achieved by successive crossings, seeking to obtain tetraploid hybrids by crossing diploid and triploid cultivars. For this purpose, special attention is focused on the diploid germplasm (AA), since it concentrates the largest number of desirable traits, such as parthenocarpy, good number of hands, long fingers (fruits), well-formed bunches, low plant height and resistance to pests and

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Abbreviations: TTC, 2,3,5-Triphenyltetrazolium chloride; **RH,** relative humidity; **H_3BO_3,** boric acid; **KNO_3,** potassium nitrate; **$Ca(NO_3)2.4H_2O$,** calcium nitrate tetrahydrate; **$MgSO_4.7H_2O$,** magnesium sulfate heptahydrate; **HCl,** hydrochloric acid.

diseases (Amorim et al., 2013). Therefore, it is essential to know the pollen fertility of diploid hybrids, since they will be used as male genitors in genetic improvement programs. Analysis of the viability of pollen supplies an indication of the male fertility, allowing better targeting and more security in the crosses carried out, increasing the efficiency of obtaining desirable hybrids. The viability of pollen can be determined using direct methods, such as inducing germination *in vitro* (Jiang et al., 2009; Alcaraz et al., 2011; Rodriguez-Enriquez et al., 2013) and *in vivo* (Dane and Ekici, 2011; Fakhim et al., 2011), or by indirect methods based on cytological parameters, such as staining (Burke et al., 2007; Abdelgadir et al., 2012; Bhat et al., 2012; Ćalić et al., 2013). Pollen vigor is a prerequisite for the fertilization and development of seeds. In the research of tissue culture, pollen germination rate and the length of pollen tube were usually used as indicators of pollen vigor (Ma et al., 2012). Therefore, studies of pollen growth in culture media, the determination of the best collection times and the histochemical analysis of pollen are fundamental in reproductive biology and genetic improvement to obtain the highest pollen viability and develop new hybrids with traits of agronomic interest. Additionally, other factors that can influence pollen germination are the nutritional state of the plant, season of the year, time of day, temperature and pH of the culture medium (Boavida and McCormick, 2007; Soares et al., 2008; Daher et al., 2009; Distefano et al., 2012; Rodriguez-Enriquez et al., 2013).

The pH of germination medium is an important factor controlling pollen germination and pollen tube development in different plant species (Qiu et al., 2005; Acar et al., 2010; Boavida and McCormick, 2007; Zaman et al., 2009; Conner, 2011; Rodriguez-Enriquez et al., 2013). Also, it has influence on the nutrient availability, plant regulators amount and the degree of agar solidification. Several studies have showed data on the pH ranges and optimum values for pollen germination and pollen tube in different species (Munzuroglu et al., 2003; Qiu et al., 2005; Zaman, 2009). However, for banana, little information exists about the effect of pH on *in vitro* pollen germination (Soares et al., 2008). In any pollination method, pollen viability is considered satisfactory when between 50 and 70% of the pollen germinate. In the field, the pollen of some species only become viable several hours after release and remain so only for short time, during which the temperature must be moderate, the humidity high and the light intensity sufficient for germination to occur. As the pollen grains get older, the percentage of germination and the average of tube length decline. Even when pollen appears otherwise unviable, the presence of some vigorous tubes indicates that they can produce effective fructification, albeit with low germination percentage (Scorza and Sherman, 1995). Knowledge of the best time to collect pollen to obtain high viability is important to produce hybrid seeds, particularly of species where artificial hybridization is possible. Although, studies have been published on methods to assess the viability of banana pollen, to date there are no reports to our knowledge of the influence of the time of pollen collection along the day and the year on hybridization efficiency.

Therefore, this study aimed to determine the best moment to collect pollen from diploid banana plant genotypes with high viability using *in vitro* germination and staining with 2,3,5-triphenyltetrazolium chloride (TTC). Pollen was collected at 5 time points (8 am, 10 am, 12 pm, 2 pm and 4 pm) during two seasons of the year (Winter and Summer).

MATERIALS AND METHODS

Materials

The study was carried out in the experimental field of the Embrapa Cassava and Fruits, located at 12° 40' S. latitude and 39° 06' W. longitude, in Cruz das Almas, Bahia, Brazil. The climate in the region, according to the Köppen classification, is a transition between Am and Aw zones, with average annual rainfall of 1,143 mm, average temperature of 24.28°C and relative humidity (RH) of 60.47%. The experiments were conducted separately in the winter (June, 22.1°C, 87, 6% RH) and summer (December, 25.3°C, 76,2% RH).

Plant material

We used pollen from six improved banana plant diploid genotypes (AA), generated by the banana improvement program of Embrapa Cassava and Fruits (Table 1). The pollen grains were collected from flowers in anthesis, removed from the same bract, at five different times of the day (8 am, 10 am, 12 pm, 2 pm and 4 pm). Then pollen grains collected at each time point were placed *in vitro* in a culture medium for germination and tube growth evaluation.

Methods

In vitro pollen germination and pollen tube length evaluation

For *in vitro* germination assays, pollen grains that were not subjected to any aseptic processes were inoculated in Petri dishes (9 cm diameter and 1 cm height) containing 35 mL of the culture medium proposed by Soares et al. (2008). Briefly, the medium is composed of 15% sucrose, 0.01% boric acid, 0.01% potassium nitrate, 0.03% calcium nitrate and 0.02% magnesium sulfate, solidified with 0.8% agar and adjusted at pH of 5.8 or 7.0 before autoclaving at 121°C for 20 min. For each Petri dish we used a pool composed of pollen from five flowers of each genotype. After inoculation, the pollen grains were kept at 27±1°C in the dark for 24 h. The germinated pollen grains were then counted, and the pollen tube length was measured using a binocular stereomicroscope. The experimental design was completely randomized using a 6 × 5 × 2 factorial arrangement (genotype × collection time × pH), with eight replicates. To calculate the *in vitro* germination percentage, 100 randomly selected pollen grains were counted. Regarding pollen tube length, five randomly selected pollen tubes from each replicate were measured (representing a total of 40 tubes per genotype). The pollen grain was considered germinated when its pollen tube diameter was equal to or larger than the pollen itself (Tuinstra and Wedel, 2000).

Table 1. Banana diploid genotypes (AA) used in this study and their respective parents.

Improved diploid genotype	Female parent	Male parent
013018-01	Malaccensis (W)	Sinwobogi (C)
042052-04	M 53 (H)	Kumburgh (C)
050012-02	M 61 (H)	Lidi (C)
088079-01	Malaccensis (W) x Madang (C)	Tuu Gia (C) x Calcutta 4 (W)
089087-01	Malaccensis (W) x Sinwobogi (C)	Calcutta 4 (W) x Heva (C)
091087-01	Borneo (W) x Guyod (C)	Calcutta 4 (W) x Heva (C)

W = Wild; C = Cultivar; H = Hybrid, with unknown parents.

Histochemical analysis

The histochemical analysis of the pollen was performed by staining them with 2,3,5-triphenyltetrazolium chloride (TTC) diluted to 1% in Tris buffer (HCl 0.15 M, pH 7.8). The TTC is an enzymatic test used to detect the dehydrogenase enzyme activity of cells. Pollen grains removed from three anthers of flowers collected from the same bract were distributed on glass slides and stained with a drop of the stain solution and covered with a slip. Observations of the number of viable and unviable pollen grains for each genotype were carried out 2 h after preparation of the slides because TTC requires a time interval for enzymatic reaction to occur. To obtain a good representativeness of the viability of the pollen grains, 100 pollen grains/slide/genotype were counted (three replicates each, for a total of 300 pollen grains) using an optical microscope. Results are expressed in percentage. Pollen grains stained by TTC in light red or dark red were counted as viable, while noncoloured were classified as unviable (Duro et al., 2013). The experimental design was completely randomized in a 6 × 5 factorial scheme (genotype × collection time) with three replicates each.

Data analysis

Percentages obtained were transformed to arc sin ($\sqrt{x/100}$) before the statistical analysis. To assess the relationship of *in vitro* germination and the histochemical test results, we applied analysis of variance, while we used the Tukey test to compare the means at 5% probability. To complement the statistical analysis we used regression analysis to choose mathematical models with the best fit, according to the highest values of the coefficient of determination (R^2) and the F-test, both at 5% significance. We used the SAS System, version 9.2 (SAS Institute, 2010) for all statistical analyses.

RESULTS

In vitro pollen germination and pollen tube length evaluation

The data obtained for the *in vitro* germination indicated a significant effect ($p<0.001$) of all the factors studied taken independently (genotype, collection time and pH), as well as a significant effect for the interaction between those factors and the two seasons (winter and summer) (Tables 2 and 3). Regarding the *in vitro* germination assay, percentage was higher in the summer than in the winter (Table 1). For all genotypes, the germination rate was in general highest for the pollen collected at 8 am and placed in the culture medium with pH 7.0 whatever the season. Among the banana diploids studied, genotype 089087-01 presented the highest germination rates, greater than 90%, when the flowers were collected at 8 am and pollen grains were placed in culture medium with pH 7.0, for both winter and summer season (Figure 1a to 1b). On the other hand, genotype 050012-02 presented the lowest germination percentages at all collection times, both in summer and winter (Figure 1c). Pollen collected in summer grew longer tubes than those collected in the winter (Table 3). Diploid 042052-04 presented the longest tube lengths, with averages of 4.80 mm in summer and 3.42 mm in winter (Figure 1d). Pollen tube growth was always longer for pollen collected at 8 am with pH 7.0 medium, except for the genotype 050012-2 where pollen tube was longer in pH 5.8 medium.

Histochemical analysis

Data obtained by histochemical staining with TTC are in agreement with data obtained on *in vitro* germination test. Indeed, genotype 089087-01 stood out, with 91.33 and 86.00% viable pollen from flowers collected at 8 am in the Summer and Winter, respectively (Table 4, Figure 1e). On the other hand, diploid 050012-02 presented the lowest viability values at all collection times, in both seasons (Figure 1f). Regardless the season for all genotypes, pollen grains collected at 8 am exhibited the highest viability values. The regression analysis showed that in both seasons, the germination rate, tube length and pollen viability, as determined by histochemistry, were negatively influenced by the time lapse from anthesis (Figures 2 and 3). There was a linear relation for germination rate, tube length and histochemistry result (y) and collection times (x), with the highest averages obtained at 8 am and the lowest at 4 pm. This behavior can be explained by the fact that the pollen grains collected at 8 am were at their peak of physiological development (anthesis), after which these values declined during the day.

DISCUSSION

In vitro germination in culture medium is a technique that simulates the conditions of the style-stigma, inducing

Table 2. Percentage of *in vitro* pollen germination from diploid banana plants (AA), collected at different times of the day in two different seasons (winter and summer).

Collection time	Genotypes											
	013018-01		042052-04		050012-02		088079-01		089087-01		091087-01	
	pH 5.8	pH 7.0	pH 5.8	pH 7.0	pH 5.8	pH 7.0	pH 5.8	pH 7.0	pH 5.8	pH 7.0	pH 5.8	pH 7.0
Winter												
8 am	46.36^{aB}	69.41^{aA}	55.42^{aB}	78.79^{aA}	14.99^{aB}	23.94^{aA}	31.22^{aB}	48.50^{aA}	55.47^{aB}	90.75^{aA}	49.44^{aB}	80.05^{aA}
10 am	37.19^{bB}	47.84^{bA}	40.25^{bB}	53.11^{bA}	11.21^{aB}	18.77^{aA}	20.75^{bB}	29.45^{bA}	37.42^{bB}	62.53^{bA}	32.77^{bB}	44.58^{bA}
12 am	29.55^{cB}	39.13^{cA}	33.40^{cB}	44.51^{cA}	6.56^{bB}	12.21^{bA}	15.53^{bB}	21.98^{cA}	33.02^{bB}	49.34^{cA}	26.91^{cB}	35.99^{cA}
2 pm	22.41^{dB}	28.84^{dA}	27.62^{dA}	32.76^{dA}	3.66^{bA}	7.49^{cA}	10.74^{cA}	15.39^{dA}	28.07^{cB}	40.07^{dA}	25.59^{cB}	35.11^{cA}
4 pm	15.96^{eA}	18.99^{eA}	18.77^{eA}	22.29^{eA}	2.95^{bA}	5.95^{cA}	10.42^{cA}	11.64^{dA}	17.34^{dB}	25.06^{eA}	15.35^{dA}	20.35^{dA}
CV (%)	10.62											
Summer												
8 am	53.75^{aB}	76.50^{aA}	64.37^{aB}	84.50^{aA}	32.87^{aB}	41.50^{aA}	39.12^{aB}	52.37^{aA}	66.00^{aB}	96.37^{aA}	51.50^{aB}	76.37^{aA}
10 am	44.00^{bB}	52.62^{bA}	51.87^{bB}	66.37^{bA}	29.00^{aB}	40.12^{aA}	29.75^{bB}	43.25^{bA}	51.37^{bB}	77.00^{bA}	40.87^{bB}	59.50^{bA}
12 am	34.25^{cB}	44.87^{cA}	43.50^{cB}	52.37^{cA}	21.37^{bB}	29.62^{bA}	28.62^{bB}	38.50^{bA}	44.25^{cB}	57.87^{cA}	31.50^{cB}	51.50^{cA}
2 pm	31.00^{cB}	36.25^{dA}	31.87^{dB}	41.75^{dA}	10.50^{cB}	23.50^{cA}	24.12^{bB}	30.00^{cA}	37.00^{dB}	51.87^{dA}	25.00^{dB}	41.00^{dA}
4 pm	21.87^{dB}	29.50^{eA}	24.87^{eB}	35.00^{eA}	5.50^{cB}	19.75^{cA}	8.87^{cB}	25.75^{cA}	29.75^{eB}	39.50^{eA}	21.87^{dB}	35.12^{eA}
CV (%)	10.26											

Means followed by the same lower-case letters in the column and upper-case letters in the row within the same factor do not differ from each other by the Tukey test at 5% probability. Pollen germination was assessed in culture media described by Soares et al. (2008). Two different pH were tested (pH: 5.8 and 7.0).

Table 3. Measurement of pollen tube length (mm) from diploid banana plants (AA) collected at different times of the day in two different seasons (winter and summer).

Collection time	Genotype											
	013018-01		042052-04		050012-02		088079-01		089087-01		091087-01	
	pH 5.8	pH 7.0	pH 5.8	pH 7.0	pH 5.8	pH 7.0	pH 5.8	pH 7.0	pH 5.8	pH 7.0	pH 5.8	pH 7.0
Winter												
8 am	2.17^{aB}	2.87^{aA}	2.68^{aB}	3.42^{aA}	1.01^{bA}	0.91^{aA}	0.91^{aA}	1.08^{aA}	1.16^{aA}	1.32^{aA}	1.03^{abB}	1.80^{abA}
10 am	1.90^{aB}	2.71^{aA}	2.14^{bB}	2.71^{bA}	1.05^{bA}	0.81^{aB}	0.81^{aA}	0.77^{bA}	0.77^{bA}	0.92^{bA}	1.09^{aB}	2.03^{aA}
12 am	1.35^{bB}	2.08^{bA}	1.62^{cB}	2.24^{cA}	1.37^{aA}	0.80^{aB}	0.80^{aA}	0.70^{bA}	0.85^{bA}	0.77^{bcA}	0.80^{bcB}	1.72^{bA}
2 pm	1.04^{cB}	1.43^{cA}	1.07^{dA}	1.14^{dA}	0.90^{bcA}	0.67^{aB}	0.75^{aA}	0.64^{bA}	0.68^{bA}	0.58^{cA}	0.74^{bcB}	1.08^{cA}
4 pm	0.75^{cB}	1.37^{cA}	0.96^{dB}	1.40^{dA}	0.69^{cA}	0.75^{aA}	0.67^{aA}	0.65^{bA}	0.61^{bA}	0.55^{cA}	0.70^{cB}	1.04^{cA}
CV (%)	38.82											
Summer												
8 am	2.50^{aB}	3.83^{aA}	3.36^{aB}	4.80^{aA}	1.66^{aA}	1.19^{aB}	1.19^{aB}	1.61^{aA}	1.29^{aB}	1.76^{aA}	1.29^{aB}	2.86^{aA}
10 am	2.56^{aB}	3.19^{bA}	2.56^{bB}	3.62^{bA}	1.63^{aA}	1.14^{aB}	1.14^{aA}	1.10^{bA}	1.14^{aA}	1.33^{bA}	1.30^{aB}	2.67^{aA}
12 am	1.94^{bB}	2.56^{cA}	2.15^{cB}	2.70^{cA}	1.66^{aA}	1.20^{aB}	1.20^{aA}	1.04^{bA}	0.80^{bB}	1.26^{bA}	1.06^{abB}	2.25^{bA}
2 pm	1.30^{dB}	2.10^{dA}	1.55^{dB}	2.37^{cA}	1.27^{bA}	0.95^{aB}	0.95^{aA}	0.88^{bA}	0.57^{bB}	0.85^{cA}	0.92^{bB}	1.51^{cA}
4 pm	1.03^{dB}	1.95^{dA}	1.50^{dB}	2.00^{dA}	0.87^{dA}	0.88^{aA}	0.88^{aA}	0.80^{bA}	0.51^{bB}	0.83^{cA}	0.90^{bB}	1.40^{cA}
CV (%)	32.10											

Means followed by the same lower-case letters in the column and upper-case letters in the row within the same factor do not differ from each other by the Tukey test at 5% probability. Pollen germination was assessed in culture media described by Soares et al. (2008). Two different pH were tested (pH: 5.8 and 7.0).

germination and pollen tube growth. Each species requires a specific protocol of culture medium to obtain adequate pollen grain germination (Soares et al., 2013). The pH of the culture medium considered ideal for *in vitro*

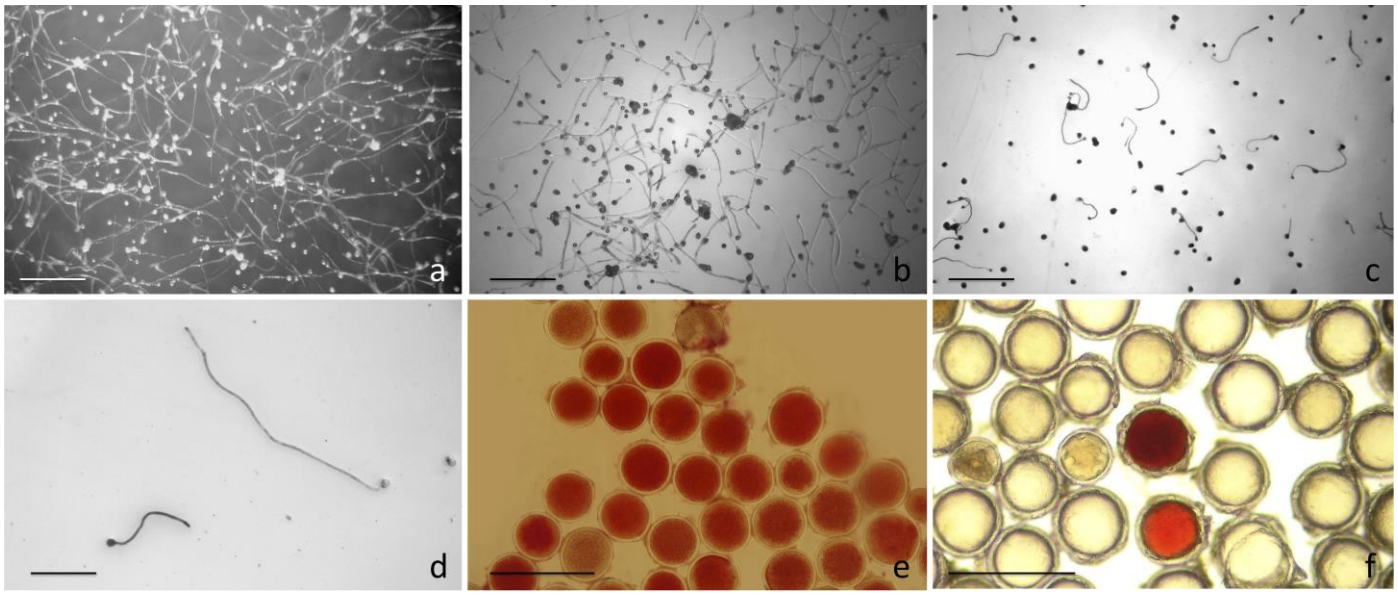

Figure 1. Viability of the pollen from diploid banana plants. Germination of pollen from genotype 089087-01 in culture medium with pH 7.0, from flowers collected at 8 am in summer (a) and winter (b). Germination of pollen from genotype 050012-02 in culture medium with pH 5.8, from flowers collected at 8 am in winter (c). Greater length of the pollen tube of genotype 042052-04 in culture medium with pH 7.0, from flowers collected at 8 am in summer (d). Histochemical test with TTC on genotype 089087-01, evidencing the high viability of the pollen when collected at 8 am in summer (e). Histochemical test with TTC on genotype 050012-02, evidencing the low viability of the pollen when collected at 8 am in winter (f). Bars: a-d = 2 mm; e-f = 200 µm.

Table 4. Percentage of pollen viability from diploid banana plants (AA) determined by the histochemical test with TTC (2,3,5-triphenyltetrazolium chloride) at 1% both in Winter and Summer.

Genotype	8 am	10 am	12 am	2 pm	4 pm
Winter					
013018-01	57.83^{dA}	55.20^{cA}	42.73^{cB}	37.30^{cB}	38.27^{bB}
042052-04	75.00^{bA}	63.13^{bB}	56.10^{bB}	46.43^{bC}	42.73^{aC}
050012-02	34.13^{fA}	24.43^{dB}	17.90^{dC}	17.80^{eC}	11.70^{dD}
088079-01	46.60^{eB}	51.43^{cA}	41.43^{cB}	33.03^{cD}	34.70^{cD}
089087-01	86.00^{aA}	73.43^{aB}	63.00^{aC}	51.30^{aD}	46.03^{aD}
091087-01	65.90^{cA}	55.80^{cB}	48.70^{cB}	41.73^{bC}	41.90^{aC}
CV(%)			4.26		
Summer					
013018-01	77.33^{bA}	67.00^{bB}	60.33^{bB}	53.00^{aC}	41.67^{aC}
042052-04	82.33^{bA}	75.00^{bA}	59.67^{bB}	49.00^{aC}	44.00^{aC}
050012-02	45.00^{dA}	35.00^{dB}	27.33^{bC}	24.67^{cC}	19.00^{cC}
088079-01	60.66^{cA}	52.33^{cB}	46.00^{cB}	36.00^{bC}	32.67^{bC}
089087-01	91.33^{aA}	84.00^{aA}	70.67^{aB}	58.00^{aC}	48.67^{aD}
091087-01	79.67^{bA}	68.00^{bB}	55.33^{bC}	41.00^{bD}	39.00^{bD}
CV (%)			7.25		

Means followed by the same lower-case letters in the column and upper-case letters in the row within the same factor do not differ from each other by the Tukey test at 5% probability.

pollen germination and pollen tube growth of different species of plants is situated near neutrality or in alkaline condition (Acar et al., 2010, Conner, 2011; Boavida and McCormick, 2007; Rodriguez-Enriquez et al., 2013). In banana, Soares et al. (2008) observed that the culture medium with pH 7.0 promoted greater pollen germination percentage. In this study, we showed that pH 5.8 of the culture medium negatively affected the *in vitro* pollen

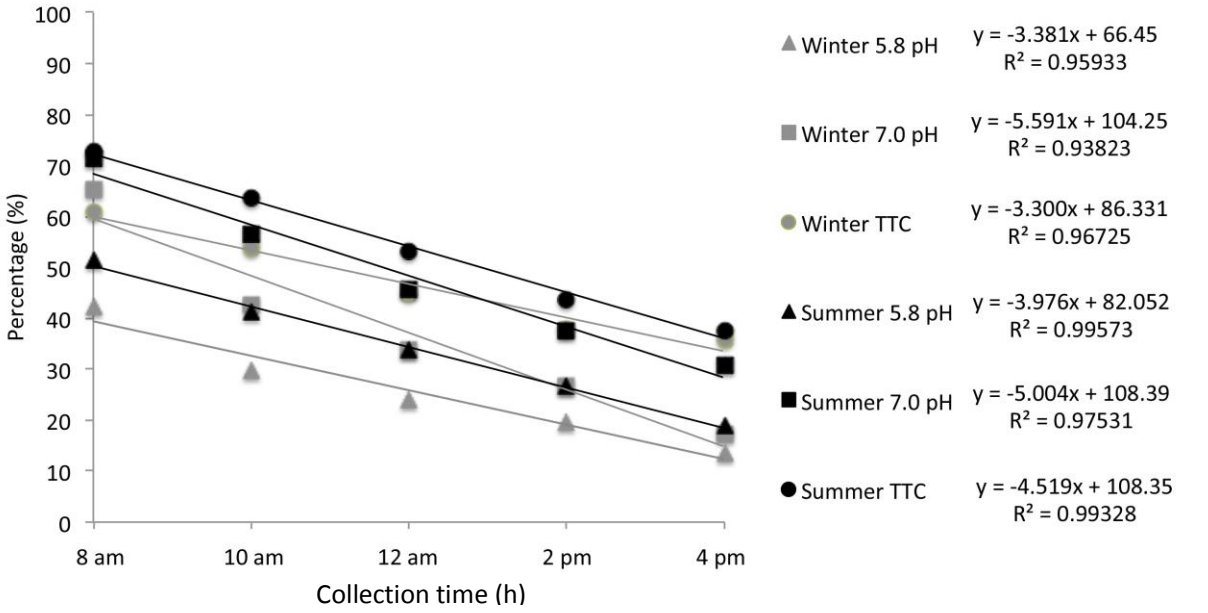

Figure 2. Percentage of pollen germination and pollen viability estimated by histochemistry, from diploid banana plants, calculated at 5 different time points during a day and for two seasons of the year (winter and summer). For pollen germination, tests were performed *in vitro* on culture media adjusted at pH: 5.8 and 7.0 as described by Soares et al. (2008).

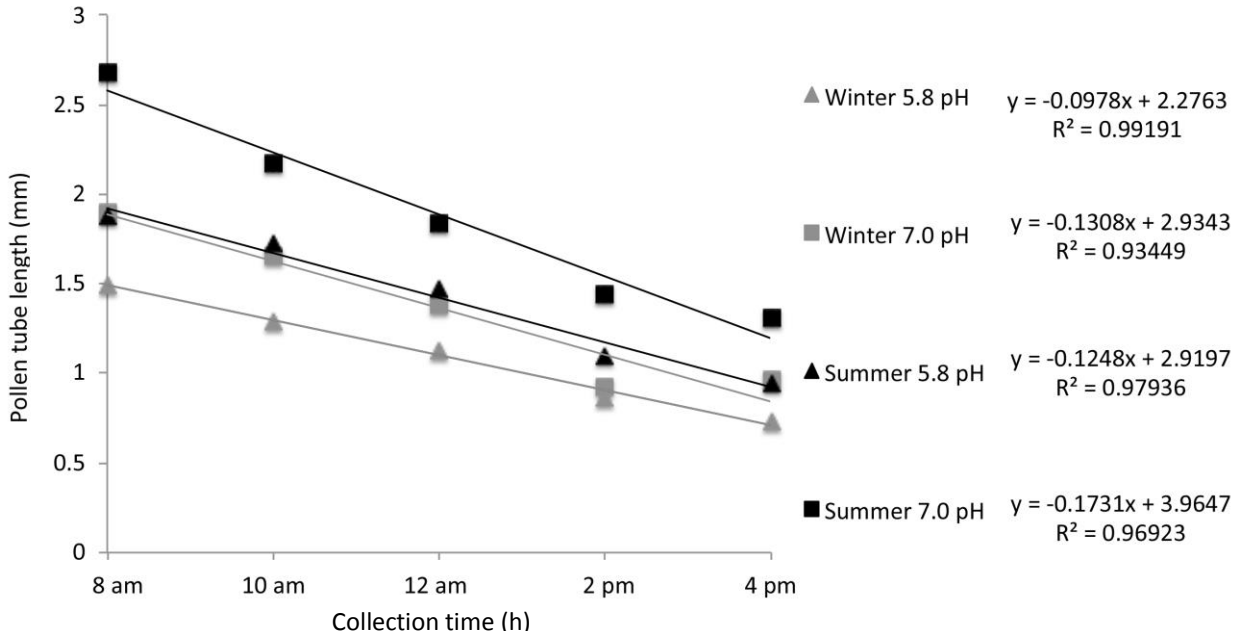

Figure 3. Pollen tube length (mm) of diploid banana plants expressed at five different time points during a day and for two seasons of the year (winter and summer). For pollen tube length measurements, tests were performed *in vitro* on culture media adjusted at pH: 5.8 and 7.0 as described by Soares et al. (2008).

germination and pollen tube growth when compared to pH 7.0, except for 050012-02 genotype which had highest pollen tube length in pH 5.8. Probably these differences in pH medium preferences for pollen tube growth are due to the great influence of the genetic variation among genotypes. Similarly, Sharafi et al. (2011) observed that pollen germination and pollen tube growth varied according to species, cultivar and method

that may have been used. Furthermore, Kakani et al. (2005) concluded that the differences observed in *in vitro* pollen germination and pollen tube growth of 12 cotton cultivars (*Gossypium hirsutum* L.) were reflections of the variability of the cultivars. In *Arabidopsis thaliana* (L.) Heynh., Costa et al. (2013) observed differences in the percentage of *in vitro* pollen germination and pollen tube growth in relation to the culture media. Other factors influenced *in vitro* pollen germination as incubation time after placing the pollen on the plates, development state of the flowers when collected and storage conditions (Qiu et al., 2005; Rodriguez-Enriquez et al., 2012). The maturation of pollen is one of the development stages of the life cycle of plants. *In vitro* germination does not occur within the anther, but the pollen must be ready to germinate soon after the anther's dehiscence (Lin and Dickinson, 1984). Therefore, hybridization will be more effective when using pollen collected at the optimal time of the day.

Veiga et al. (2012), studying corn pollen collected at different time points (9 am, 2 pm and 4 pm), found that the maize pollen collected in the morning presented higher germination rates than those collected in the afternoon. The results presented here show that the time of pollen collection in banana along the day affects its viability. We indeed, observed a maximum of pollen viability (Figure 2) when pollen was collected at 8 am whatever the genotypes tested. Loss of pollen viability in function of time after flower opening was also observed by Souza et al. (2002) in yellow passion fruit plants using histochemical analyses by Alexander (1980) and Lugol (Johansen, 1940), although the index remained high, above 75%, even 24 h after anthesis.

Judd et al. (1999) also established that the viability of pollen can be altered by variations in humidity and temperature, and this can be tested by means of germination capacity, enzyme activity and presence of a cytoplasm. According to the authors, this variation can be species-specific: for example, while the pollen of some grasses is only viable for a few minutes or hours, the pollen of other species can remain viable for many years if stored properly. Dusi et al. (2010) also found that the external environmental conditions influence the *in vitro* germination of pollen of *Brachiaria* spp. accessions. According to them, the pollen grains gathered during the rainy season or the day after a rainstorm are unable to germinate. According to Zonia et al. (2002), pollen tubes tend to rupture because of the increase of hydrostatic pressure and low cell wall resistance. This allows a rapid inflow of water in the pollen, causing the loss of soluble substances and ions in the cytoplasm, known as "imbibition damage" (Loguercio, 2002). Some of our preliminary studies (data not presented) go in the same direction because we observed inefficient germination and intense eclosion after rainy days. For these reasons, we did not collect pollen on rainy days. *In vitro* germination rates and viability according to histochemical analysis are directly related (Scorza and Sherman, 1995). In general, the results obtained in the present study are in agreement with that statement, given that our pollen germination data indicated a significantly lower rate in pH 5.8 than that observed when using staining materials. Other authors also have reported that the observations from histochemical tests lead to overestimation of the pollen germination percentage, while *in vitro* test results cause underestimation (Sutyemez, 2011; Coser et al., 2012).

Although, the culture medium used in the *in vitro* pollen germination may simulate the pistil conditions, it is impossible for *in vitro* method to display all conditions that affect pollen tube growth *in vivo* (Hedhly et al., 2005). The assessment of viability using TTC is based on the color change of the tissues in the presence of a salt solution of 2,3,5-triphenyltetrazolium chloride, which is reduced by dehydrogenase respiratory enzymes in live tissues, resulting in a red carmine-colored compound called formazan (Beyhan and Serdan, 2008). The TTC test has been used to estimate the pollen viability in many species (Kelen and Demirtas, 2003; Huang et al., 2004; Kang et al., 2009; Soares et al., 2013). Several authors argued that the TTC test is a reliable estimative of pollen viability, showing similar results to *in vitro* germination tests (Bolat and Pirlak, 1999; Huang et al., 2004). In addition, the TTC is widely used because it is relatively fast and easy method. Abdelgadir et al. (2012) observed that only TCC staining was able to differentiate viable and unviable pollen in *Jatropha curcas* L. (Euphorbiaceae). Similarly, Huang et al. (2004), who studied the viability of *Leymus chinensis* (Trin.) Tzvelev pollen using the TTC stain was able to distinguish the relative decline of the pollen viability percentage after anthesis. Knowledge of the adequate moment to collect pollen is essential for genetic improvement programs involving banana plants, mainly to obtain more efficient crosses. In the present study, we observed that the best time to collect pollen for hybridization is at 8 am on the day of floral anthesis and that *in vitro* germination and growth of the pollen tube are much better in summer time. The pollen collected at that time showed high viability in the test with TTC staining and the highest *in vitro* germination percentage, both in the winter and summer.

Conflict of interests

The authors have not declared any conflict of interests.

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REFERENCES

Abdelgadir HA, Johnson SD, Van Staden J (2012). Pollen viability, pollen germination and pollen tube growth in the biofuel seed crop *Jatropha curcas* (Euphorbiaceae). S. Afr. J. Bot. 79(1):132-139.

Acar I, Arpaci S, Eti S (2010). Pollen susceptibility of *Pistacia* species to different pH medium. Afr. J. Agric. Res. 5(14):1830-1836.

Alcaraz ML, Montserrat M, Hormaza JI (2011). *In vitro* pollen germination in avocado (*Persea americana* Mill.): Optimization of the method and effect of temperature. Sci. Hortic. 130(1): 152-156.

Alexander MP (1980). A versatile stain for pollen, fungi, yeast and bacteria. Stain Technol. 55(1):13-18.

Amorim EP, Santos-Serejo JA, Amorim VBO, Ferreira CF, Silva SO (2013). Banana Breeding at Embrapa Cassava and Fruits. Acta Hortic. 986: 171-176.

Beyhan N, Serdar U (2008) Assessment of pollen viability and germinability in some European chestnut genotypes (*Castanea sativa* L.). Hort. Sci. 35:171–178.

Bhat ZA, Dhillon WS, Shafi RHS, Rather JA, Mir AH, Shafi W, Rashid R, Bhat JA, Rather TR, Wani TA (2012). Influence of storage temperature on viability and *in vitro* germination capacity of pear (*Pyrus spp*.) pollen. J. Agric. Sci. 4(11): 128-135.

Boavida LC, McCormick S (2007). Temperature as a determinant factor for increased and reproducible *in vitro* pollen germination in *Arabidopsis thaliana*. The Plant J. 52(3):570-582.

Burke IC, Wilcut JW, Allen NS (2007). Viability and *in vitro* germination of johnsongrass (*Sorghum halepense*) pollen. Weed Technol. 21(1):23-29.

Ćalić D, Devrnja N, Kostić I, Kostić M (2013). Pollen morphology, viability, and germination of Prunus domestica cv. Požegača. Sci. Hortic. 155: 118-122.

Conner PJ (2011). Optimization of *in vitro* pecan pollen germination. Hortscience 46(4):571-576. 2011.

Coser SM, Fontes MMP, Ferreira MFS (2012). Assessment of pollen viability in guava genotypes. Acta Hortic. 959: 140-144.

Costa ML, Pereira LG, Coimbra S (2013). Growth media induces variation in cell wall associated gene expression in *Arabidopsis thaliana* pollen tube. Plants. 2:429-440.

Daher FB, Chebli Y, Geitmann A (2009). Optimization of conditions for germination of cold-stored *Arabidopsis thaliana* pollen. Plant Cell Rep. 28(3):347-357.

Dane F, Ekici N (2011). Pollen tube growth of *Paeonia tenuifolia* L. (Paeoniaceae) *in vitro* and *in vivo*. Bangladesh J. Bot. 40 (3): 93-95.

Distefano G, Hedhlyb A, Casasa G, Malfaa SL, Herreroc M, Gentilea A (2012). Male-female interaction and temperature variation affect pollen performance in *Citrus*. Sci. Hortic. 140(1):1-7.

Duro A, Piccione V, Zampino D (2013) Air quality biomonitoring through pollen viability of Fabaceae. Environ. Monit. Assess. 185:3803–3817.

Dusi DMA, Alves ER, Willemse MTM, Falcão R, Valle CB, Carneiro VTC (2010). Toward *in vitro* fertilization in *Brachiaria* spp. Sex. Plant Reprod. 23(3):1870-197.

Dutta SK, Srivastav M, Rymbai H, Chaudhary R, Singh AK, Dubey AK, Lal K (2013). Pollen–pistil interaction studies in mango (*Mangifera indica* L.) cultivars. Sci. Hortic. 160:213-221.

Edlund AF, Swanson R, Preuss D (2004). Pollen and Stigma Structure and Function: The Role of Diversity in Pollination. The Plant Cell, 16(1):84-97.

Fakhim RSH, Hajilou J, Zaare NF (2011). Pollen germination and pistil performance in several Iranian peach cultivars. Int. J. AgriScience, 1(3):71-77.

Hedhly A, Hormaza JI, Herrero M (2005). Influence of genotype-temperature interaction on pollen performance. J. Evol . Biol. 18(6): 1494-1502.

Heslop-Harrison Y (2000). Control gates and micro-ecology: The pollen–stigma interaction in perspective. Ann. Bot. 85:5-13.

Huang Z, Zhu J, Mu X, Lin J (2004). Pollen dispersion, pollen viability and pistil receptivity in *Leymus chinensis*. Ann Bot. 93(3):295-301.

Jiang B, Shen Z, Shen J, Yuda D, Sheng X, LU H (2009). Germination and growth of sponge gourd (*Luffa cylindrica*) pollen tubes and FTIR analysis of the pollen tube wall. Sci. Hortic. 122(4):638-644.

Johansen DA (1940) Plant microtechnique. New York: McGraw-Hill Book

Judd WS, Campbell PS, Qellogg EA, Stevens PS (1999). Plant sytstematics: a phylogenetic approach. Sinaver

Kakani VG, Reddy KR, Koti S, Wallace TP, Prasad PVV, Reddy VR, Zhao D (2005). Differences *in vitro* pollen germination and pollen tube growth of cotton cultivars in response to high temperature. Ann. Bot. 96(1):59-67.

Kang HG, Bae TW, Jeong OC, Sun HJ, Lim PO, Lee HY (2009). Evaluation of viability, shedding pattern, and longevity of pollen from genetically modified (GM) herbicide-tolerant and wild-type Zoysiagrass (*Zoysia japonica* Steud.). J. Plant Biol. 52(6): 630-634.

Kelen M, Demirtas I (2003). Pollen viability, germinationcapability and pollen production level of some grape varieties (*Vitis vinifera* L.). Acta Physiol. Plant. 25(3):229-233.

Lin JJ, Dickinson DB (1984). Ability of pollen to germinate prior to anthesis and effect of desiccation on germination. Plant Physiol. 74(3):746-748.

Loguercio LL (2002). Pollen treatment in high osmotic potential: a simple tool for *in vitro* preservation and manipulation of viability in gametophytic populations. Braz. J. Plant Physiol. 14(1): 65-70.

Losada JM, Herrero M (2014). Glycoprotein composition along the pistil of *Malus* x *domestica* and the modulation of pollen tube growth. BMC Plant Biol. 14(1):1-14.

Ma W, Wang J, Hu J, Guan Y, Li Y, Zheng Y (2012). Relation between changes in polyamine, protective enzyme activity and pollen vigor of tobacco in different flowering stages. Afr. J. Agric. Res. 7(40):5491-5497.

Madureira HC, Pereira TNS, Cunha M, Klein DE (2012). Histological analysis of pollen-pistil interactions in sour passion fruit plants *(Passiflora edulis* Sims). Biocell, 36(2): 83-90.

Munzuroglu O, Obek E, Gecgil H (2003). Effects of simulated acid rain on the pollen germination and pollen tube growth of apple (*Malus sylvestris* Miller cv. Golden). Acta Biol. Hung. 54(1):95-103.

Qiu DL, Liu XH, Guo SZ (2005). Effects of simulated acid rain on fertility of litchi. J. Environ. Sci. 17(6):1034-1037.

Rodriguez-Enriquez MJ, Mehdi S, Dickinson HG, Grant-Downton RT (2013). A novel method for efficient *in vitro* germination and tube growth of *Arabidopsis thaliana* pollen. New Phytol. 197(2):668-679.

Sas Institute (2010). SAS user's guide: statistics, version 9.2 Cary: SAS Institute.

Scorza R, Sherman WB (1995). Peaches, in: Janik J, Moore JN (Eds.) Fruit breeding. New York, John & Sons. pp. 325-440.

Sharafi Y, Motallebbi-Azar AR, Bahmani A (2011). *In vitro* pollen germination, pollen tube growth and longevity in some genotypes of loquat (*Eriobotria japonica* Lindl.). Afr. J. Biotech. 10:8064-8069.

Soares TL, Jesus ON, Souza EH, Santos-Serejo JA, Oliveira EJ (2013). Morphology and viability of pollen grains from passion fruit species (*Passiflora* spp.). Acta Bot. Bas. 27(4):779-787.

Soares TL, Silva SO, Costa MAPC, Santos-Serejo JA, Souza AS, Lino LSM, Souza EH, Jesus ON (2008). *In vitro* germination and viability of pollen grains of banana diploids. Crop Breed. Appl. Biotechnol. 8(2):111-118.

Souza MM, Pereira TNS, Martins ER (2002). Microsporogenesis and microgametogenesis with relation to flower bud and anther size, and pollen viability in yellow passion fruit (*Passiflora edulis* Sims f. *flavicarpa* Degener). Ciênc. Agrotec. 26(6):1209-1217.

Sutyemez M (2011). Pollen quality, quantity and fruit set of some selfcompatible and self-incompatible cherry cultivars with artificial pollination. Afr. J. Biotechnol. 10(17):3380-3386.

Tuinstra MR, Wedel J (2000). Estimation of pollen viability in grain sorghum. Crop Sci. 40(4): 968-970.

Veiga POA, Pinho RGV, Pinho EVRV, Veiga AD, Oliveira KC, Diniz RP (2012). Culture media for germination of pollen grains of maize. Rev. Agrar. 5(17):206-211.

Zaman MR (2009). Effect of pH on *in vitro* pollen germination of fourteen cultivated and wild species of cucurbit. J. Bio-Sci. 17:129-133.

Zonia L, Cordeiro S, Tupy J, Feijo J (2002). Oscillatory chloride efflux at the pollen tube apex has a role in growth and cell volume regulation and is targeted by inositol 3,4,5,6-tetrakisphosphate. The Plant Cell. 14(9):2233-2249.

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Bioprospecting of yeasts for amylase production in solid state fermentation and evaluation of the catalytic properties of enzymatic extracts

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Profiling microorganisms with potential for amylase production in low cost culture media has been widely recognized due to its broad applicability. The aim of this study was to select yeast strains with potential to produce amylolytic enzymes by solid state fermentation. Fifty-four (54) strains were assessed and three exhibited ability to produce amylases: *Candida parapsilosis* with 14.68 U/mL (146.8 U/g substrate); *Rhodotorula mucilaginosa* with 25.0 U/mL (250 U/g substrate), and *Candida glabrata* with 25.39 U/mL (253.9 U/g substrate), in solid state fermentation, for 120 h at 28°C, using wheat bran with 70% moisture. The enzymes exhibited maximum activity at a pH of 7.0 and at 60°C. Amylases demonstrated satisfactory structural stability, maintaining their catalytic activity after 1 h at 50°C. All enzymes were ethanol tolerant and retained more than 70% of their original activities in 15% ethanol solution. Corn starch was efficiently hydrolyzed by enzymes and the extracts produced by *C. parapsilosis* and *C. glabrata* exhibited dextrinizing activity, while those produced by *R. mucilaginosa* exhibited saccharifying activity.

Key words: *Candida parapsilosis*, *Candida glabrata, Rhodotorula mucilaginosa,* dextrinizing and saccharifying activity.

INTRODUCTION

The improvement of ethanol production processes and the development of new biotechnological techniques to use plant-based polysaccharides are important to ensure mankind's supply of energy requirements (Scott et al., 2013).

In Brazil, ethanol is produced by fermenting monosaccharides from the hydrolysis of sucrose by *Saccharomyces cerevisiae* yeast, which metabolizes under appropriate

conditions, releasing ethyl alcohol as the main product of fermentation (Basso et al., 2008). To meet the demand for this biofuel, several studies have focused on devising efficient mechanisms to hydrolyze starch and cellulose, thereby obtaining free glucose that can be fermented into ethanol by *S. cerevisiae*. In this regard, enzymes that catalyze the hydrolysis of these polymers are considered very important in the global energy scenario (Gupta et al., 2003; Sahnoun et al., 2012).

The main polysaccharide of plants is starch, which consists of glucose residues linked by glycosidic bonds (α-1,4 and α-1,6) that are found mainly in rice, corn, wheat, cassava and potato (Zeeman et al., 2010), all of them widely consumed and produced in different Brazilian regions. Starch can be hydrolyzed by chemical or enzymatic techniques and the action of different enzymes is necessary for its efficient conversion into products of lower molecular weight, such as dextrins, maltose and glucose. Enzymatic hydrolysis has some advantages compared to chemical methods, the biocatalysts act under mild conditions of pH and temperature, reducing energy consumption, equipments corrosion and eliminates neutralization steps. However, specificity of enzymatic catalysis can be considered as the main advantage of the enzymes use, preventing the formation of undesirable byproducts commonly observed in reactions by chemical catalysis (Gupta et al., 2003; Sivaramakrishnan et al., 2006).

Amylases account for 25-33% of the international enzyme market and are used in numerous industrial processes that require the partial or total hydrolysis of starch (Özdemir et al., 2011). Endoamylolytic and debranching enzymes (α-amylases and isoamylases, respectively) reduce the degree of polymerization of the starch molecule, producing linear glucose-based dextrins. These enzymes are employed in the starch liquefaction process. Exoamylases are used in subsequent steps of the enzymatic hydrolysis of starch. These enzymes hydrolyze the dextrins from the liquefaction, producing maltose (β-amylases) or glucose (amyloglucosidase, α-glucosidases and glucoamylases) syrups that are used in the food, beverage and biofuel industries (Silva et al., 2005; Sivaramakrishnan et al., 2006; Özdemir et al., 2011).

The use of yeast to produce enzymes offers certain advantages, such as a moderate temperature for microbial growth, high metabolic diversity and rapid cell growth, which results in shorter fermentation cycles and easy adaptation to different cultivation conditions (Kato et al., 2007). The use of agroindustrial wastes as substrate in solid state fermentation reduces enzyme production costs and contributes to minimize environmental problems caused by the agroindustry (Singhania et al., 2009). These advantages explain the search for microbial strains that exhibit significant enzyme production in low-cost fermentation processes (Alves-Prado et al., 2010). The use of agroindustrial wastes for enzymes production can become economically viable for the application of these biocatalysts in large scale, considering that one of the major problems in the enzymes utilization in industrial processes is the high cost of the microbial culture media, about 30-40% of the cost enzyme production (Romero et al., 2007; García-Martínez et al., 2010).

Therefore, the aims of this study were to select yeast strains having the potential to produce amylolytic enzymes by solid state fermentation, characterize them biochemically, and evaluate their catalytic properties.

MATERIALS AND METHODS

Microorganisms

The present study analyzed 54 yeast strains supplied by the organization RECOL – Rede Centro-Oeste de Leveduras (Central-Western Yeast Network, Brazil), and one strain of *Saccharomyces cerevisiae* supplied by the São Fernando sugar and alcohol mill in the municipality of Dourados, MS, Brazil. The strains from RECOL were isolated in natural and industrial environments such as fruit from the Cerrado, poultry litter and sugarcane must.

Inoculum preparation

The strains were cultivated in test tubes containing 5 mL of YEPD medium (yeast extract 1%, peptone 2%, glucose 2% and agar 1.5%) for 48 h at 28°C. The yeast suspension was obtained by scraping off the surface of the medium using 3 mL of nutrient solution (0.5% ammonium sulfate, 0.5% magnesium sulfate heptahydrate and 0.5% ammonium nitrate). Yeast was inoculated in the substrate by transferring 3 mL of the microbial suspension (at 10^6 cells/mL) to Erlenmeyer flasks containing wheat bran.

Selection of strains for amylase production by solid state fermentation

The yeasts were cultivated in solid state in Erlenmeyer flasks (250 mL) containing 5 g of wheat bran (ground to 2-3 mm size) with 70% moisture content (mass of dry substrate per volume), using the above described nutrient solution to moisten the substrate. Prior to the inoculation of microorganisms, all the material was autoclaved at 121°C for 20 min. After inoculation, the Erlenmeyer flasks were stored at 28°C for 120 h. Due to the high number of strains evaluated in this study, the cultivation parameters (substrate, moisture, temperature and fermentation time) were fixed near values described as optimal, for amylases production by different fungal strains, in previous works as a comparison parameter

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(Ellaiah et al., 2002; Bhatti et al., 2007). All the assays were performed in duplicate.

Enzyme extraction

Enzymes were extracted by adding 50 mL of distilled water to the flasks containing the ferment medium. The flasks were kept under agitation at 150 rpm for 1 h, after which they were filtered through Whatman paper (n.1) and centrifuged at 3000x *g* for 10 min at 25°C. The supernatant was used for the enzyme assays.

Determination of amylase activity

The enzyme activity was determined by adding 0.1 mL of enzymatic extract to 0.9 mL of sodium acetate buffer (0.1 M, pH 5.0, 1% corn starch). After 10 min of reaction at 50°C, the reducing sugar released was quantified by the 3,5-dinitrosalicilic acid method (DNS) (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of the product per minute of reaction.

Identification of selected strains

High level amylase-producing strains were subjected to molecular identification (DNA sequencing). Genomic DNA was extracted from three days cultures grown in yeast-malt extract agar at 25°C (Sampaio et al., 2001). The D1/D2 domains of the 26S rRNA gene were amplified using the universal primer pair NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3'). This region is generally used as the molecular marker for yeast identification (Kurtzman and Robnett, 1998; Schoch et al., 2012). PCR conditions were as follows: 96°C for 3 min, 35 cycles at 96°C for 30 s, 61°C for 45 s, and an extension step at 72°C for 1 min (Rodrigues et al., 2009). The expected size of amplicons (500-600 base pairs) were checked after electrophoresis in 1% agarose gel supplemented with GelRed® (Biotium). Amplicons were purified and then quantified in NanoDrop® 2000 (Thermo Scientific). Sanger cycle sequencing reactions were performed with BigDye Terminator v. 3.1 (Life Technologies), using 20 ng of amplicon template and the same primers used in the PCR. Both forward and reverse sequences were generated in ABI3330xl (Life Technologies) and assembled in BioEdit v.7.0.5.3 (Hall, 1999). Consensus sequences were queried in the NCBI – GenBank and CBS (Centraalbureau voor Schimmelcultures) databases. We adopted the criterion used by Kurtzman and Robnett (1998), and query sequences that showed 99% identity with those deposited in the database were considered conspecific.

Biochemical characterization of the amylases produced

Effect of pH and temperature

The optimum pH was determined by measuring the enzyme activity at 50°C with different pH conditions using McIlvaine buffer (0.1 M), due to its wide buffering range (from 3.0 to 8.0). The optimum temperature was defined by measuring the enzyme activity in different temperature conditions (from 30 to 75°C) at the respective optimal pH of each enzyme. The pH stability of enzymes was assessed by incubating them at 25°C for 24 h at different levels of pH. The following buffers were used: McIlvaine 0.1 M (3.0 - 8.0); Tris-HCl 0.1 M (8.0 to 8.5) and glycine-NaOH 0.1 M (8.5 - 10.5). Thermostability was assessed by incubating the enzymes for 1 h at different temperatures (30 to 75°C). The residual activity was determined under optimum conditions of pH and temperature for enzymes (Leite et al., 2008).

Effect of ethanol on enzyme activities

Enzyme activity was quantified by adding different concentrations of ethanol (0 to 30%) to the reaction mixture. The assays were performed at 50°C in McIlvaine buffer 0.1 M (pH 7.0, 1% corn starch) (Leite et al., 2008).

Catalytic potential for different sources of starch

Enzyme assays were performed using potato, corn, wheat and cassava starch (1%) as enzyme substrate, vegetal cheap sources and available in Brazil. The enzymatic reactions were performed in McIlvaine buffer 0.1 M (pH 7.0). The sugar released was quantified using the DNS method (Miller, 1959).

Dextrinization potential of enzymatic extracts

Dextrinizing activity was assessed using corn starch (1%) as enzyme substrate in McIlvaine buffers 0.1 M (pH 7.0) and the iodometric methods described by Fuwa (1954) and Pongsawadi and Yagisawa (1987). The reaction mix contained 0.1 mL of extract enzymes in 0.3 mL of buffer solution containing starch. After 10 min at 60°C, the reaction was stopped by adding 4 mL of HCl solution 0.2 M. Finally, 0.5 mL of reactive iodine and 10 mL of distilled water were added. The absorbance was quantified at 700 nm. One unit of activity was defined as the amount of enzyme required to reduce the intensity of the blue of the starch iodine complex by 10% per minute of reaction.

Saccharification potential of enzymatic extracts

Saccharifying activity was assessed using corn starch (1%) as enzyme substrate in McIlvaine buffer 0.1 M (pH 7.0) and employing the glucose-oxidase/peroxidase method (Bergmeyer and Bernt, 1974). The reaction mix contained 0.1 mL of the extract enzymatic in 0.4 mL of buffer solution containing starch. After 10 min at 60°C, the reaction was stopped in an ice bath. The glucose released was quantified using an enzymatic colorimetric kit (*Glicose-PP Analisa*). The absorbance was quantified at 505 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of glucose per minute of reaction.

RESULTS AND DISCUSSION

Selection and identification of strains with potential for amylase production

To determine their potential for amylase production by solid state fermentation, the 54 yeast strains were grown for 120 h at 28°C, using wheat bran with 70% moisture. Wheat bran was chosen as the substrate for the selection of promising strains due to its complexity nutritional, favoring microbial growth and the production of different fungal amylases; fact described by several authors in previous works (Ellaiah et al., 2002; Bhatti et al., 2007).

Table 1. Production of amylase by solid state fermentation for 120 hours at 28°C, using wheat bran (70% moisture content) as substrate.

Yeast	Collection site	Amylase activity (U/mL)
01	Cereja do Rio Grande**	5.07 ± 0.08
29*	Uvaia**	14.68 ± 0.5
30*	Uvaia	25.00 ± 0.16
37*	Pêssego do Mato**	14.10 ± 0.44
39	Acerola**	5.39 ± 0.19
41	Acerola	11.22 ± 0.27
43*	Pequi**	25.39 ± 0.23
44	Pequi	10.17 ± 0.27
46	Uvaia	5.54 ± 0.18
53	Sugarcane must	10.30 ± 1.25
54	Sugarcane must	11.77 ± 0.20
S. cerevisiae	Commercial strain	0.39 ± 0.05

*Strains 29 and 37 (*Candida parapsilosis*); Strain 30 (*Rhodotorula mucilaginosa*); Strain 43 (*Candida glabrata*).**Cereja do Rio Grande *(Eugenia involucrata DC);* Uvaia *(Eugenia pyriformis);* Pêssego do Mato *(Hexachlamys edulis);* Acerola *(Malpighia glabra L.);* Pequi *(Caryocar brasiliense)*.

Table 2. Molecular identification of amylase-producing yeast strains using the D1/D2 rRNA gene marker.

Yeast	Identification	Size[1]	GenBank closest relative[2]		CBS closest relative[2]	
			%	Accession #	%	Accession #
29	*Candida parapsilosis*	571	99	*Candida parapsilosis* (AB370926)	100	*Candida parapsilosis* CBS 10947
30	*Rhodotorula mucilaginosa*	571	100	*Rhodotorula mucilaginosa* ATCC 4056 (KC881063)	100	*Rhodotorula mucilaginosa* PYCC 5995
37	*Candida parapsilosis*	570	100	*Candida parapsilosis* (AB370926)	100	*Candida parapsilosis* PYCC 2545
43	*Candida glabrata*	581	100	*Candida glabrata* CBS 138 (AY198398)	100	*Candida glabrata* CBS 858

[1]In base pairs; [2]Results obtained in the databases.

Solid state fermentation was adopted aiming to lower the cost of enzymes production, considering that one of the major problems for the enzymes application in industrial scale, is the high cost of these biocatalysts, given the high price of formulated culture media (Romero et al., 2007; García-Martínez et al, 2010). In this way, the use of agroindustrial wastes for the microorganisms cultivation and enzyme production is one alternative to reduce the final cost of these biological catalysts (Singhania et al., 2009; Alves-Prado et al., 2010), especially in countries such as Brazil that have agriculture as one of the main economic activities.

Under the culture conditions used in this study, the strains that displayed the highest production of extracellular amylase were strains 29 (14.68 U/mL or 146.8 U/g substrate), 30 (25 U/mL or 250 U/g substrate), 37 (14.10 U/mL or 141 U/g substrate) and 43 (25.39 U/mL or 253.9 U/g substrate) (Table 1). These results are very promising when compared to previous results reported in the literature. Bhatti et al. (2007) cultivated the fungus *Fusarium solani* in solid state, using wheat bran as substrate, and obtained glucoamylase (about 61.35 U/g). Anto et al. (2006) reported the production of α-amylase by *Bacillus cereus* MTCC1305 (about 122 U/g), also grown in solid state. Similar results were reported by Ellaiah et al. (2002), who cultivated *Aspergillus* species in solid state. The authors obtained the maximum glucoamylase production (247 U/g), after 120 h of cultivation.

High level amylase-producing strains were selected for molecular identification. Using the criterion established by Kurtzman and Robnett (1998), our results show that the D1/D2 sequences of strains 29 and 37 were 99% identical to a sequence of *Candida parapsilosis* found in the database (Table 2). On the other hand, sequences

Table 3. Effect of pH and temperature on amylase produced by the selected yeast strains.

Yeast	Optimum pH	Optimum temperature (°C)	Stability pH	Stability temperature (°C)
Candida parapsilosis	7.0	60	5 -10	30 - 50
Rhodotorula mucilaginosa	7.0	60	3.5 - 9.5	30 - 50
Candida glabrata	7.0	60	3 - 8.5	30 -50

derived from strains 30 and 43 were 100 and 99% identical to a sequence of *Rhodotorula mucilaginosa* and *Candida glabrata,* respectively (Table 2). Reports about the use of these strains to produce enzymes of industrial interest are scarce, which underlines the importance of the present study. According to Saran et al. (2007), the prospection of microorganism producers of industrially-relevant enzymes has become increasingly important since it enables an understanding and improved application of these enzymes in industrial processes, as well as the discovery of new catalytic properties that may be used to develop new biotechnological processes.

Biochemical characterization of the amylases produced

Effect of pH and temperature

Amylases were characterized using enzymatic extracts obtained by growing the yeasts under the previously described conditions. The enzymes exhibited maximum activity at pH 7.0 and 60°C (Table 3). In general, fungal amylases have optimum pH values ranging from acidic to neutral (Gupta et al., 2003). Giannesi et al. (2006) obtained amylases from several microbial sources, exhibiting an optimum pH from 4.5 to 7.0. Rahardjo et al. (2005) reported pH 7.0 as optimal for amylases produced by *Aspergillus oryzae.* Figueira and Hirooka (2000) found optimum pH values of around 6.7 for amylases produced by the fungus *Fusarium moniliforme*. With respect to temperature, Aquino et al. (2003) reported that 60°C was the optimal temperature for α-amylase produced by *Scytalidium thermophilum*. Similar results were found by Li et al. (2007) for amylase produced by the yeast *Aureobasidium pullulans* N13d.

The enzymes were stable in a broad range of pH: *C. parapsilosis* (5 to 10); *R. mucilaginosa* (3.5 to 9.5) and *C. glabrata* (3 to 8.5). Amylases produced by selected strains were stable for 1 h at 50°C (Table 3). The enzymes exhibited higher pH and temperature-related stability than amylases produced by different microbial species. The amylase produced by *Vibrio* sp. was stable at pH 6.0 to 7.5, maintaining only 50% of its activity at pH 4.5 and 8.5. The same enzyme was stable for 30 min at 50°C (Najafi and Kembhavi, 2005). Sahnoum et al. (2012) found that the amylases produced by *Aspergillus oryzae* remained stable after 48 h at pH 5.6. However, when incubated for the same period at other levels of pH, they exhibited a considerable decrease in their catalytic potential. In the same study, the authors reported that the enzymes exhibited about 60% of residual activity after 1 h at 50°C. Gomes et al. (2005) reported a decrease of approximately 30% in the catalytic activity of amylase produced by the fungus *Thermomyces lanuginosus* when incubated for 24 h at pH 4.0 and 10.0. However, the enzyme remained stable after 1 h at 60°C.

The presence of enzymatic activity at extremes of pH and temperature confirms the high structural stability of the amylases produced by the yeasts assessed in the present study, highlighting their biotechnological potential. Previous studies reported high stability for extracellular enzymes produced by yeasts (Fossi et al., 2005; Leite et al., 2008). Enzymatic action in a wide range of pH and temperatures is advantageous for their industrial application, considering that only minor adjustments are needed in the processes (Leite et al., 2008).

Effect of ethanol on enzymatic activity

Enzymes may be exposed to alcoholic solutions in various industrial applications; therefore, the inhibition of ethanol is a trend in the study of some enzymes (Sun and Cheng, 2002). When incubated in solutions containing up to 15% ethanol, the enzyme activity was higher than 70% of the original (Figure 1). The increased catalytic potential of the amylases produced by the yeasts *C. parapsilosis* and *R. mucilaginosa* may be associated with the use of ethanol as the acceptor of intermediate glycosylation during the hydrolysis of the substrate, increasing the speed of the reaction (Villena et al., 2006).

Considering that the final concentration of ethanol in traditional fermentation processes stabilizes at around 10%, the results obtained here are very promising. Ethanol concentrations higher than 12% are harmful to the fermenting microorganism, hindering the recycling of yeast for a new fermentation cycle (Gu et al., 2001). Therefore, it can be inferred that the enzymes produced by the selected strains may be applied in alcohol production processes from amylaceous sources. Enzymatic stability in ethanol is an advantageous feature in simultaneous saccharification and fermentation (SSF) processes in which fermentable sugars released by the action of enzymes on vegetable polysaccharides are converted simultaneously into ethanol by fermenting micro-

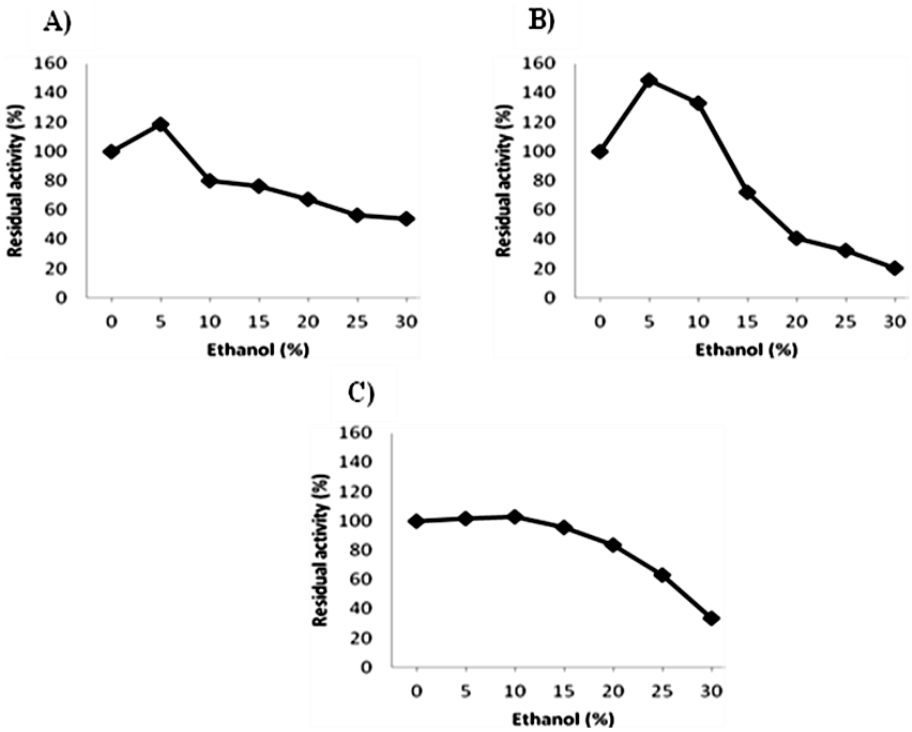

Figure 1. Effect of ethanol on enzymatic activity at pH 7.0 and 50°C. **A)** *Candida parapsilosis*. **B)** *Rhodotorula mucilaginosa*. **C)** *Candida glabrata*.

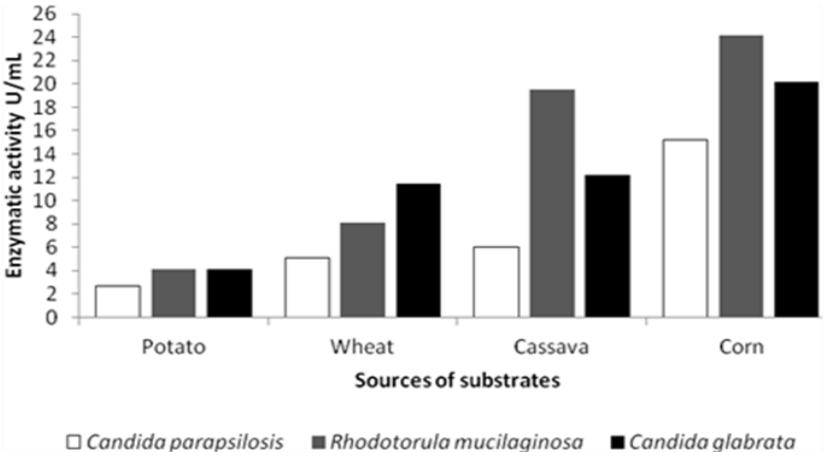

Figure 2. Catalytic potential of the enzymes on starches from different vegetable sources, quantified by the DNS method (reducing sugar).

organisms (Leite et al., 2008; Scott et al., 2013).

Enzymatic hydrolysis of starch from different sources

The action of enzymes on starch from different vegetable sources was assessed using starch extracted from potatoes, wheat, cassava and corn as substrate. These are among the main starch sources commercialized in the world, being all cultivated expressively in Brazil.

The enzymatic extracts exhibited potential to hydrolyze all the starch assessed in this study. However, the best catalytic efficiency was achieved with corn starch (Figure 2).

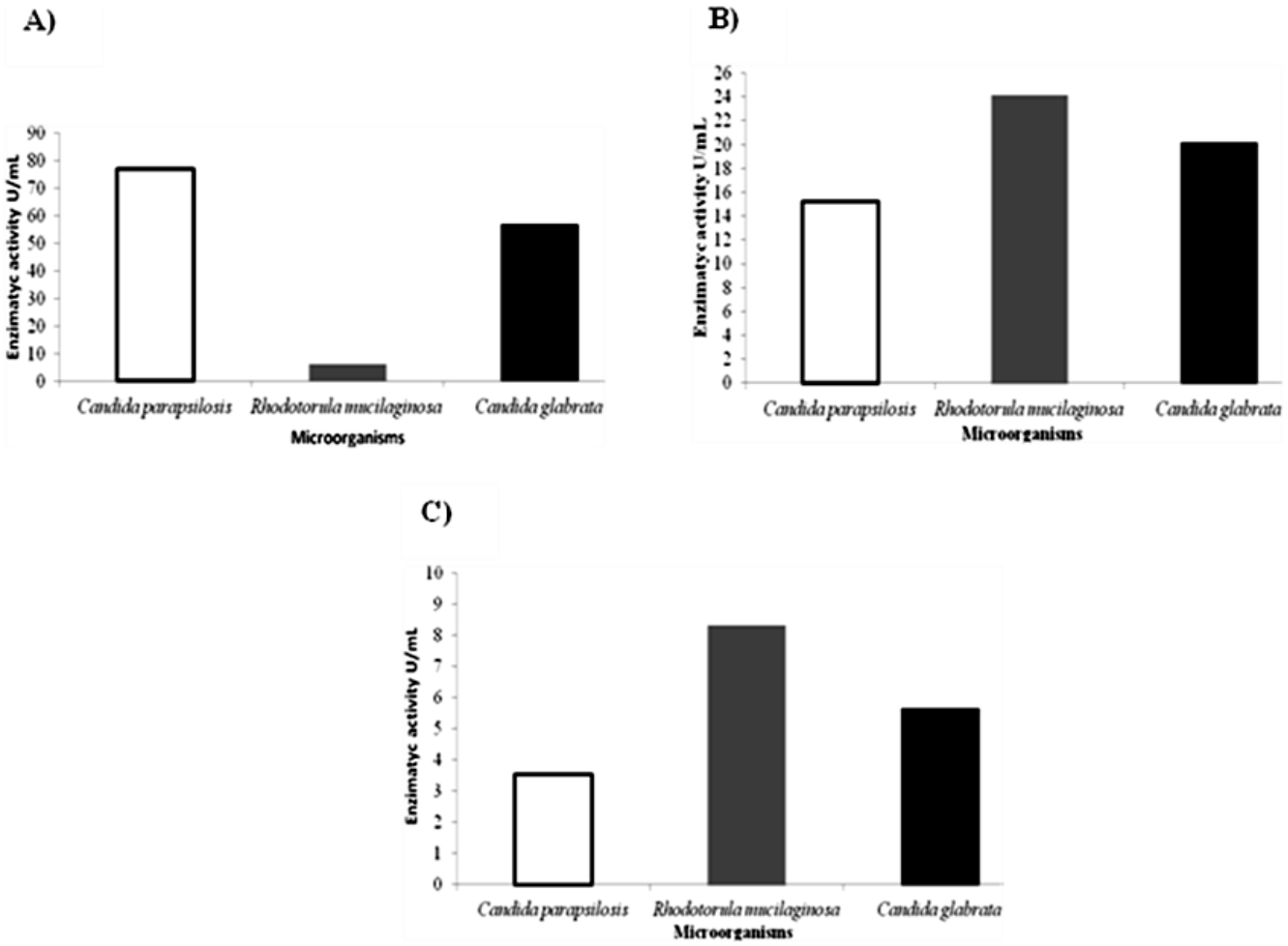

Figure 3. Enzymatic modifications of corn starch. **A)** Quantification of dextrinizing activity using the iodometric method. **B)** Quantification of sugars and reducing ends using the DNS method. **C)** Quantification of free glucose using the glucose-oxidase method.

The catalytic action of different enzymes in starch from different sources may be related to the structure and composition of the substrate molecule, particularly the content and length of the amylose chains. The structural characteristics of starch vary according to its botanical origin. The proportions of amylose and amylopectin affect the texture and architecture of the starch granule and are therefore reflected in the catalytic properties of amylases. Furthermore, the proportions of contaminants, such as lipids, proteins and minerals, also differ according to vegetable origin, and may affect enzymatic reactions (Thomas and Atwell, 1999; Tester et al., 2004).

Corn starch has a higher amount of amylose, and consequently lower levels of amylopectin, favoring enzymatic hydrolysis. The amylopectin molecule is larger than the amylose molecule and exhibits a branched structure of high molecular weight with α-1.4 and α-1.6 glycosidic bonds, which hinder the catalytic performance of amylases (Tester et al., 2004).

Dextrinization and saccharification potential of enzymatic extracts

Based on the above described results, the changes caused by each enzymatic extract in starch molecules were assessed using corn starch as substrate. The enzymes produced by *C. parapsilosis* and *C. glabrata* caused a significant reduction in the degree of polymerization of the starch molecule, which was reflected in the increase of reducing chain ends. However, the presence of free glucose was also observed after enzymatic treatment, indicating that the extracts have the synergistic action of dextrinizing and saccharifying enzymes, despite the predominance of depolymerizing potential (Figure 3). The enzyme extract produced by *R. mucilaginous* was not efficient in reducing

the polymerization of the starch molecule. However, an increase in the concentration of total reducing sugars and glucose was confirmed after enzymatic hydrolysis. Therefore, it can be concluded that this enzymatic extract exhibits predominantly saccharification potential (Figure 3).

The enzymatic hydrolysis of starch includes liquefaction and saccharification. Endoamylases and debranching enzymes such as α-amylase and isoamylase are used during liquefaction, drastically reducing the degree of polymerization of starch and releasing dextrins. During saccharification, dextrins are hydrolyzed by exoamylases to produce maltose (β-amylase) or glucose (amyloglucosidase, α-glucosidase and glucoamylases) syrup (Van Der Maarel et al., 2002). Thus, the results obtained in this study indicate that the enzymatic extracts of *C. parapsilosis* and *C. glabrata* exhibited catalytic exoamylase and endoamylase activity, while the extract of *R. mucilaginosa* exhibited mainly exoamylase activity. The scientific literature describes similar results. Silva et al. (2005) reported the dextrinizing and saccharifying activity of enzymatic extract produced by the filamentous fungus *Rhizomucor pusillus*. The production of exoamylases has been described for the yeasts *Aureobasidium pullulans* (Li et al., 2007) and *Schizosaccharomyces pombe* (Okuyama et al., 2005). Furthermore, predominantly dextrinizing activity has been reported for several species of *Aspergillus*, such as *A. flavus*, *A. niger* and *A. oryzae* (Shafique et al., 2009; Sahnoun et al., 2012).

Conclusions

Our results indicate that the evaluated yeast strains have potential for amylase production in low cost culture media. The enzymes exhibited high structural stability, maintaining their catalytic potential at different levels of pH, temperatures and ethanol concentrations. In view of the described characteristics, we highlight the biotechnological potential of these enzymes and intend to apply them in processes to obtain ethanol from starch sources in future studies.

Conflict of interests

The authors did not declare any conflict of interest.

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REFERENCES

Alves-Prado HF, Pavezzi FC, Leite RSR, Oliveira VM, Sette LD, Da-Silva R (2010). Screening and production study of microbial xylanase producers from Brazilian Cerrado. Appl. Biochem. Biotechnol. 161(1-8): 333-346.

Anto H, Trivedi U, Patel K (2006). Alpha amylase production by *Bacillus cereus* MTCC 1305 using solid state fermentation. Food Technol. Biotechnol. 44(2):241-245.

Aquino ACMM, Jorge JA, Terenzi HF, Polizeli MLTM (2003). Studies on a thermostable α-amylase from thermophilic fungus *Scytalidium thermophilum*. Appl. Microbiol. Biotechnol. 61(4):323-328.

Basso LC, Amorim HV, Oliveira AJ, Lopes ML (2008). Yeast selection for fuel ethanol production in Brazil. FEMS Yeast Res. 8(7):1155-1163.

Bergmeyer HU, Bernt E (1974). Methods of Enzymatic Analysis. New York: Verlag Chimie-Academic Press.

Bhatti HN, Rashid MH, Nawaz R, Asgher M, Perveen R, Jabbar A (2007). Optimization of media for enhanced glucoamylase production in solid state fermentation by *Fusarium solani*. Food Technol. Biotechnol. 45(1):51-56.

Ellaiah P, Adinarayana K, Bhavani Y, Padmaja P, Srinivasulu B (2002). Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated *Aspergillus* species. Process Biochem. 38(4):615-620.

Figueira ELZ, Hirooka EY (2000). Culture medium for amylase production by toxigenic fungi. Braz. Arch. Biol. Technol. 43(5):461-467.

Fossi BT, Tavea F, Ndjouenkeu R (2005). Production and partial characterization of a thermostable amylase from ascomycetes yeast strain isolated from starchy soils. Afr. J. Biotechnol. 4(1):14-18.

Fuwa H (1954). A new method for microdetermination of amylase activity by the use of amylose as the substrate. J. Biochem. 41(5): 583-603.

García-Martínez AM, Díaz A, Tejada M, Bautista J, Rodríguez B, Santa María C, Revilla E, Parrado J (2010). Enzymatic production of an organic soil biostimulant from wheat-condensed distiller solubles: Effects on soil biochemistry and biodiversity. Process Biochem. 45(7):1127-1133.

Giannesi GC, Polizeli MLTM, Terenzi HF, Jorge JA (2006). A novel α-glucosidase from *Chaetomium thermophilum* var. coprophilum that converts maltose into trehalose: purification and partial characterization of the enzymes. Process Biochem. 41(8): 1729-1735.

Gomes E, Souza SR, Grandi RP, Da-Silva R (2005). Production of thermostable glucoamylase by newly isolated *Aspergillus flavus* A1.1 and *Thermomyces lanuginosus* A13.37. Braz. J. Microbiol. 36(1):75-82.

Gu Y, Qiao M, Zhou Q, Zhou Z, Chen G (2001). Hyperproduction of alcohol using yeast fermentation in highly concentrated molasses medium. Tsinghua Sci. Technol. 6(3): 225-230.

Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003). Microbial α-amylases: a biotechnological perspective. Process Biochem. 38(11): 1599-1616.

Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41: 95-98.

Kato M, Fuchimoto J, Tanino T, Kondo A, Fukuda H, Ueda M (2007). Preparation of a whole-cell biocatalyst of mutated *Candida antarctica* lipase B (mCALB) by a yeast molecular display system and its practical properties. Appl. Microbiol. Biotechnol. 75(3): 549-555.

Kurtzman CP, Robnett CJ (1998). Identification and phylogeny of ascomycetous yeast from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie van Leeuwenhoek 73(4): 331-371.

Leite RSR, Alves-Prado HF, Cabral H, Pagnocca FC, Gomes E, Da-Silva R (2008). Production and characteristics comparison of crude β-

glucosidases produced by microorganisms *Thermoascus aurantiacus* e *Aureobasidium pullulans* in agricultural wastes. Enzyme Microb. Technol. 43(6):391-395.
Li H, Chi Z, Wang X, Duan X, Ma L, Gao L (2007). Purification and characterization of extracellular amylase from the marine yeast *Aureobasidium pullulans* N13d and its raw potato starch digestion. Enzyme Microb. Technol. 40(5):1006-1012.
Miller GL (1959). Use of dinitrosalicylic reagent for determination of reducing sugar. Anal Chem. 31(3):426-428.
Najafi MF, Kembhavi A (2005). One step purification and characterization of an extracellular α- amylase from marine *Vibrio sp*. Enzyme Microb. Technol. 36(4):535-539.
Okuyama M, Tanimoto Y, Ito T, Anzai A, Mori H, Kimura A (2005). Purification and characterization of the hyper-glycosilates extracellular α-glucosidase from *Schizosaccharomyces pombe*. Enzyme Microb. Technol. 37(5):472-480.
Özdemir S, Matpan F, Güven K, Baysal Z (2011). Production and characterization of partially purified extracellular thermostable α-amylase by *Bacillus subtilis* in submerged fermentation (SmF). Prep. Biochem. Biotechnol. 41(4):365-381.
Pongsawadi P, Yagisawa M (1987). Screening and indentification of a cyclomaltodextrin glucanotransferase-producing bacteria. J. Ferment. Technol. 65(4): 463-467.
Rahardjo YSP, Weber FJ, Haemers S, Tramper J, Rinzema A (2005). Aerial mycelia of *Aspergillus oryzae* accelerate α-amylase production in a model solid-state fermentation system. Enzyme Microb. Technol. 36(7):900-902.
Rodrigues A, Cable RN, Mueller UG, Bacci MJR, Pagnocca FC (2009). Antagonistic interactions between garden yeasts and microfungal garden pathogens of leaf-cutting ants. Antonie van Leeuwenhoek 96(3):331-342.
Romero E, Bautista J, García-Martínez AM, Cremades O, Parrado J (2007). Bioconversion of corn distiller's dried grains with solubles (CDDGS) to extracellular proteases and peptones. Process Biochem. 42(11):1492-1497.
Sahnoun M, Bejar S, Sayari A, Triki MA, Kriaa M, Kammoun R (2012). Production, purification and characterization of two α-amylase isoforms from a newly isolated *Aspergillus oryzae* strain S2. Process Biochem. 47(1):18-25.
Sampaio JP, Gadanho M, Santos S, Duarte FL, Pais C, Fonseca A, Fell JW (2001). Polyphasic taxonomy of basidiomycetous yeasts genus Rhodosporidium: *Rhodosporidium kratochvilovae* and related anamorphic species. Int. J. Syst. Evol. Microbiol. 51(2):687-697.
Saran S, Isar J, Saxena RK (2007). A modified method for the detection of microbial proteases on agar plates using tannic acid. J. Biochem. Biophys. Methods 70(4):697-699.
Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W (2012). The fungal barcoding consortium nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. Proc. Natl. Acad. Sci. 109(16):6241-6246.
Scott F, Quintero J, Morales M, Conejeros R, Cardona C, Aroca G (2013). Process design and sustainability in the production of bioethanol from lignocellulosic materials. Electron. J. Biotechnol. 16(3):1-16.
Shafique S, Bajwa R, Shafique S (2009). Screening of *Aspergillus niger* and *A. flavus* strains for extra cellular alpha-amylase activity. Pak. J. Bot. 41(2):897-905.
Silva TM, Angelis AD, Carvalho AFA, Da-Silva R, Boscolo M, Gomes E (2005). Production of saccharogenic and dextrinogenic amylases by *Rhizomucor pusillus* A13.36. J. Microbiol. 43(6): 561-568.
Singhania RR, Patel AK, Soccol CR, Pandey A (2009). Recent Advances in Solid-State Fermentation. Biochem. Eng. J. 44(1):13-18.
Sivaramakrishnan S, Gangadharan D, Nampoothiri KM, Soccol CR, Pandey A (2006). α-Amylases from microbial sources – an overview on recent developments. Food Technol. Biotechnol. 44(2):173-184.
Sun Y, Cheng J (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour. Technol. 83(1):1-11.
Tester RF, Karkalas J, Qi X (2004). Starch- composition, fine structure and architecture. J. Cereal Sci. 39(2):151-165.
Thomas DJ, Atwell WA (1999). Starches: practical guides for the food industry. Minnesota: Eagan Press Handbook. pp. 19-22.
Van Der Maarel MJEC, Van Der Veen B, Uitdehaag JCM, Leemhuis H, Dijkhuizen L (2002). Properties and applications of starch-converting enzymes of the α-amylase family. J. Biotechnol. 94(2): 137-155.
Villena MA, Iranzo JFU, Gundllapalli SB, Otero RRC, Pérez AIB (2006). Characterization of an exocellular β-glucosidase from *Debaryomyces pseudopolymorphus*. Enzyme Microb. Technol. 39(2):229-234.
Zeeman SC, Kossmann J, Smith AM (2010). Starch: its metabolism, evolution and biotechnological modification in plants. Annu. Rev. Plant Biol. 61:209-234.

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Full Length Research Paper

Antibacterial activity of watermelon (*Citrullus lanatus*) seed against selected microorganisms

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This study was aimed at evaluating the effect of extraction methods on the antibacterial activity of *Citrullus lanatus* seed extract. *C. lanatus* (watermelon) is a popular fruit consumed all over the world. Three solvents were used for the extraction process: chloroform, methanol and distilled water while two extraction conditions- cold extraction and Soxhlet extraction (coded as hot in this study) were employed. Antibacterial activity of the seed extracts was determined by agar well diffusion method. The seed extracts were tested against clinical isolates including *Staphylococcus* sp., *Escherichia coli, Proteus* sp., *Klebsiella* sp. and a type *Pseudomonas aeruginosa* (ATCC 27853). It was observed that the cold methanol extracts had the highest antibacterial effect on *Staphylococcus* sp. followed by hot methanol extract while cold chloroform extract showed no antibacterial activity. In the presence of *P. aeruginosa* only the hot methanol and chloroform extracts showed significant antibacterial potentials ($p \leq 0.05$). Also, saponins which have been implicated in antimicrobial activity were found to be present in moderate and high concentrations in the hot and cold methanol extracts respectively. Results of this study reveal that the kind of solvent employed as well as the conditions for extraction (cold maceration and Soxhlet extraction) influenced the efficacy of the extract against specific test organisms. Furthermore, the presence of saponins may have influence the relatively high zone of inhibition recorded with cold and hot methanol extracts against some of the test organisms.

Key words: Watermelon seed, antibacterial, Soxhlet extraction, cold maceration, solvents.

INTRODUCTION

Citrullus lanatus commonly called watermelon is a popular fruit in many parts of the world and it is notable for its high water content and attractive look. The fruit comes in various shapes, sizes and rind pattern (Wehner, 2008). Although the seed of watermelon is often discarded as waste; it contains various amounts of carbohydrate, phenol, flavonoids, protein, fibre, phosphorus and iron (Varghese et al., 2013). Proximate analysis of the seed as reported by Oyeleke et al. (2012) revealed very high fat content (47.9%) followed by protein (27.4%) and

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carbohydrate (9.9%). Traditionally, the seed of *C. lanatus* is said to be medicinal because it can relieve inflammation/ irritation; causes increased passing of urine and gives tonic effects (Okunrobo et al., 2012; Varghese et al., 2013).

Extraction of medicinal plants is carried out using various methods including maceration, infusion, decoction, percolation, hot continuous extraction (Soxhlet extraction), thermal desorption, surfactant mediated extraction, counter-current extraction, accelerated solvent extraction, pressurized liquid extraction amongst others. Advanced extraction methods include extraction, ultrasonication assisted extraction, supercritical fluid extraction, microwave-assisted extraction and supercritical fluid extraction (Gupta et al., 2012; Handa, 2008). Most of the information available on the antibacterial activity of *C. lanatus* seed have made it clear that cold maceration is popularly used for extraction of *C. lanatus* plant parts (Okunrobo et al., 2012; Omigie and Agoreyo, 2014; Oseni and Okoye, 2013); and in few cases Soxhlet extraction (Meena and Patni, 2008; Oyeleke et al., 2012). This has brought about the need to compare extraction methods in order to determine which method will yield the highest antibacterial effect on the organisms. The aim of this study was to examine the effect of extraction solvents and extraction conditions on the antibacterial potential of the seed extracts of *C. lanatus.*

MATERIALS AND METHODS

Collection and preparation of seed material

Twenty five watermelon fruits were purchased from Omu-aran market, Kwara State, Nigeria. The fruits were washed and cut open to obtain the seeds. The seeds obtained were washed and air-dried for two days; then pulverized using mortar and pestle under aseptic conditions and ground to powder using a blender (Waring commercial blender, Model No. HGB2WTS3). Powdered seed material were then weighed and kept in air-tight containers until further usage.

Preparation of crude extracts

Cold extraction by maceration and Soxhlet extraction were carried out on the seed material using three solvents- chloroform, methanol and distilled water- in order of increasing polarity.

Cold extraction: The powdered seed material was subjected to successive solvent extraction using chloroform, methanol and distilled water in the increasing order of polarity. A total of 50 g each of dried seed powder was extracted first in 150 ml of chloroform in a conical flask and placed in an orbital shaker for 72 h at 90 rpm. This was done in triplicate. The extracts obtained were filtered using Whattman filter paper and were evaporated to dryness using a rotary evaporator and water bath at 40-50°C then stored at 0-4°C in air-tight containers for further use (Varghese et al., 2013). The residue was allowed to air-dry and subsequently extracted with 150 ml of methanol following the same procedure. Lastly, the air-dried residues were extracted with 150 ml of distilled water also following the method but for 24 h.

Soxhlet extraction: The powdered seed material was also subjected to successive solvent extraction using chloroform, methanol and distilled water in the increasing order of polarity. The Soxhlet apparatus was used in this case. A total of 50 g each of dried seed powder was extracted in three thimbles (triplicate) of the Soxhlet apparatus, first in 150 ml each of chloroform and allowed to stand for 6 h. The extracts obtained were evaporated to dryness using a rotary evaporator and water bath at 40-50°C, and stored at 0-4°C in air-tight container for further use (Varghese et al., 2013). The residue was allowed to air-dry and extracted with 150 ml of methanol following the same method. Distilled water was not used for Soxhlet extraction due to the challenges encountered. All extracts were evaporated to dryness using a water bath.

Phytochemical screening

Qualitative phytochemical screening of the various extracts was carried out according to standard procedure (Trease and Evans, 1989; Evans et al., 2002) to ascertain the qualitative composition of the seed. Phytochemicals screened include alkaloids, phenols/ tannins, saponins, steroids, flavonoids, reducing sugars and lipids.

Dilution of extract

In 1 ml of each solvent used except chloroform, 0.2 g (200 mg) of the corresponding extract was re-dissolved. The chloroform extract on the other hand was re-dissolved in a mixture of petroleum ether and methanol (8:2 v/v). Ciprofloxacin was used as positive control. 1000 mg of Ciprofloxacin was dissolved in 5 ml of distilled water.

Preparation of test specimens

Clinical isolates of *Staphylococcus* sp.*, Escherichia coli, Proteus* sp.*,* and *Klebsiella* sp. were obtained from the University of Ilorin Teaching Hospital, Ilorin, Kwara State, as well as a type strain of *P. aeruginosa* (ATCC 27853). The test organisms were sub-cultured and incubated at 37°C for 24 h on nutrient agar slant medium and were stored 0-4°C till use. They were then further sub-cultured in nutrient broth at 37°C for 24 h.

In-vitro antibacterial activity (agar well diffusion method)

Antibacterial activity of watermelon seed extracts was carried out using the agar well diffusion method as reported by Hassan et al. (2011), with some modifications. Mueller Hinton agar (LAB039, LabM Limited, UK) was prepared according to the manufacturer's direction. From each standard bacterial stock suspension [*Staphylococcus* sp. (1.5×10^5 CFU/ml); *E. coli* (2.0×10^5 CFU/ml); *P. aeruginosa* (3.4×10^5 CFU/ml)], 1 ml of the broth culture of the respective bacteria was mixed thoroughly with 20 ml of sterile molten Mueller Hinton agar (45-50°C) before pouring into sterile petri dishes and left to solidify. Furthermore, a sterile cork-borer of diameter 6.0 mm was used to bore wells in each agar plate and 50 µl of each extract was introduced into the different wells. The plates were allowed to stand for 6 h after which they were incubated for 18 to 24 h at 37°C. After incubation, the zone of inhibition for each extract and the control was measured using a meter rule and recorded.

Statistical analyses were carried out using the SPSS statistical software. The comparison of means was done using the One-Way Analysis of Variance (ANOVA). All experimental setups were in triplicate.

Table 1. Qualitative phytochemical analysis of *Citrullus lanatus* seed extracts

Phytochemicals	CCE	HCE	CME	HME	CWE
Saponins	-	-	+++	++	+
Phenols/Tannin	-	-	++	+++	-
Alkaloids	-	-	++	++	++
Steroids	+++	+++	+	++	-
Flavonoids	+++	++	+++	+++	-
Reducing sugars	++	++	+++	+++	-
Lipids	++	++	+	+	+

CCE= Cold chloroform extract; HCE= hot chloroform extract; CME= cold methanol extract; HME= hot methanol extract; CWE= cold water extract; +++= High concentration; ++= moderate concentration; += low concentration; - = absent. The solvents were evaporated to dryness.

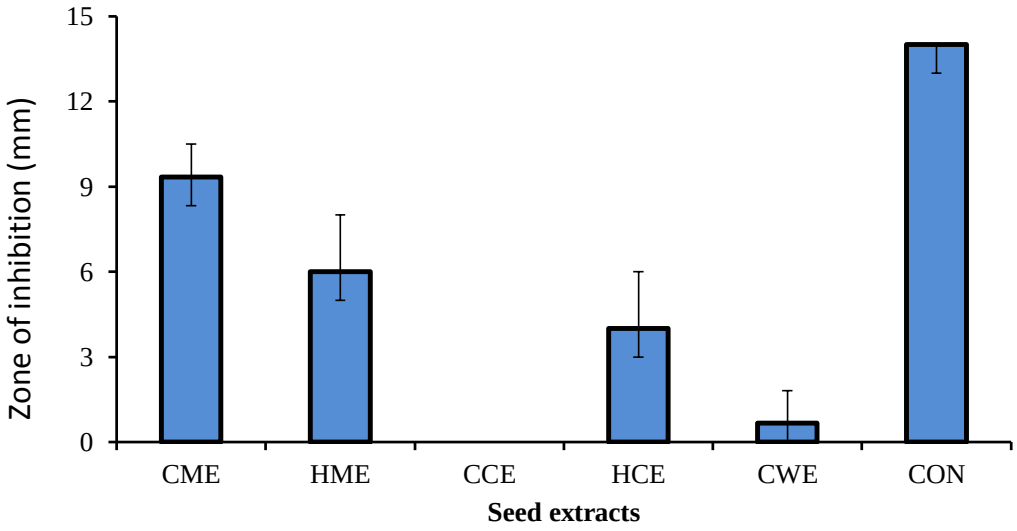

Figure 1. Antibacterial potential of the different seed extracts of the watermelon on *Staphylococcus* sp.

RESULTS

Qualitative phytochemical analysis

The various extracts of watermelon seeds were tested for different phyto-constituents including alkaloids, phenols/ tannins, saponins, steroids, flavonoids, reducing sugars and lipids using standard procedures and the results are shown in Table 1. The result reveals that the cold chloroform and hot chloroform extracts (CCE and HCE) had the same phyto-constituents; this was also the case with the cold and hot methanolic extracts (CME and HME). In addition, the methanol extracts (CME and HME) were positive for all phytochemicals screened.

Antimicrobial screening

As shown in Figure 1, the highest zone of inhibition of 9.3 mm was recorded with the cold methanol extract (CME) followed by hot methanol extract (HME) which had a zone of inhibition of 6.00 mm when administered against *Staphylococcus* sp.

Among the different extracts used for investigation, the CME and HME extracts were observed to show significantly high zones of inhibition against *Staphylococcus* sp., when compared with the other extracts ($p \leq 0.05$). Significantly low zones of inhibition were observed with the CCE and the CWE in the presence of the *Staphylococcus* sp. ($p \leq 0.05$).

When *E. coli* was used as the test bacteria, the antibacterial activity of the various extracts against *E. coli* was generally low apart from the control (Figure 2). CWE recorded a zone of inhibition of 6.0 mm while HCE had a zone of inhibition of 5.33 mm. When compared to the control antibiotic, none of the extracts used for investigation was observed to significantly inhibit the growth of *Escherichia coli*. The zones of inhibitions

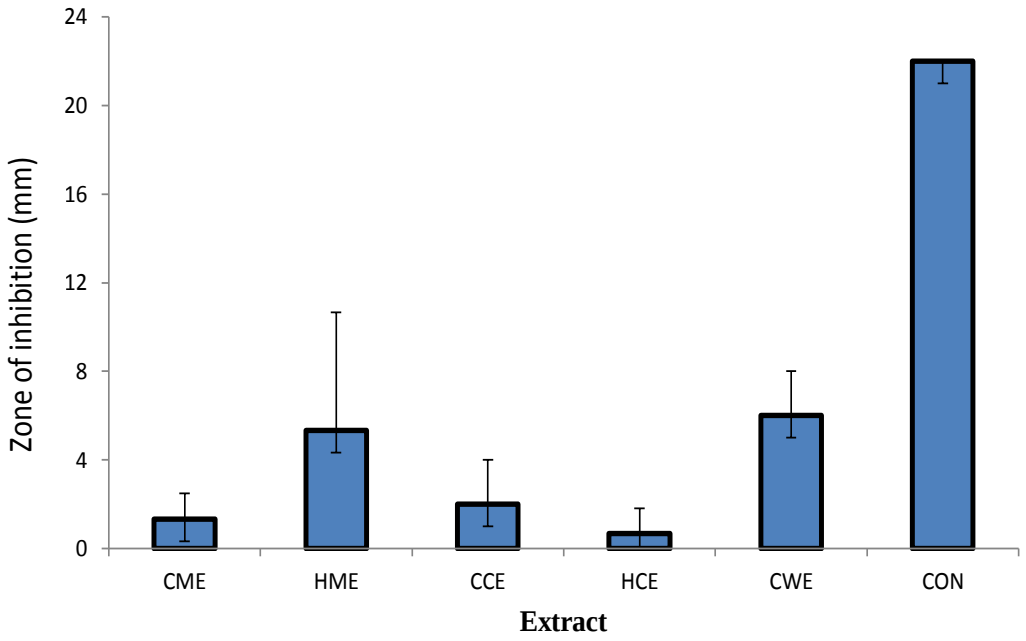

Figure 2. Antibacterial potential of the different seed extracts of the watermelon on *Escherichia coli.*

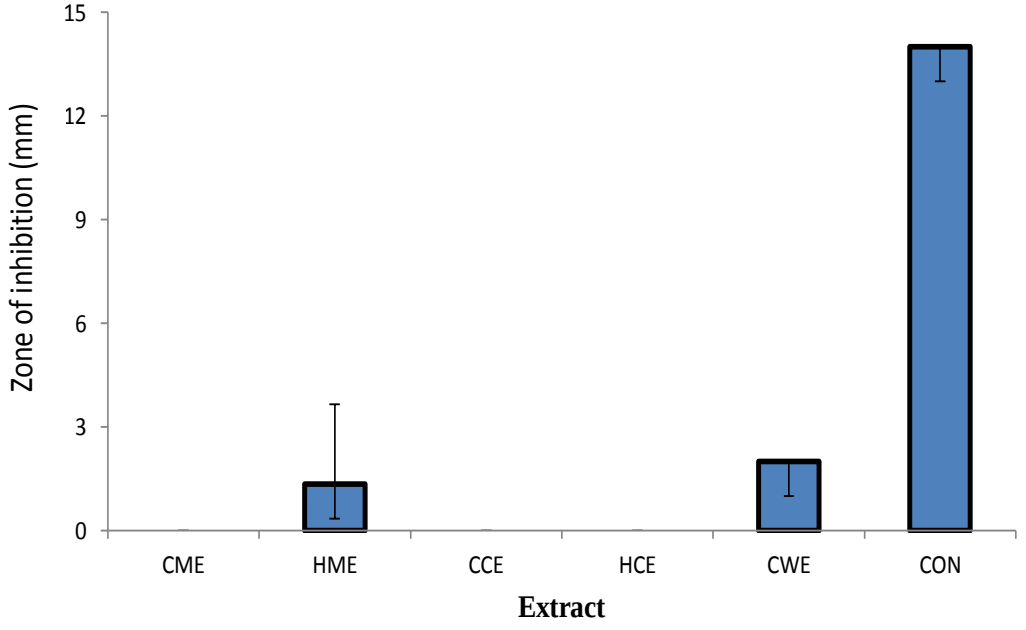

Figure 3. Antibacterial potential of the different seed extracts of the watermelon on *Proteus* sp.

observed for 'HME' and 'CWE' were however observed to be significantly higher than those of the other extracts ($p \leq 0.05$).

Figure 3 shows that CME, HCE and CCE extracts did not show any zone of inhibition when tested against *Proteus* sp. while CWE and HME recorded zones of inhibition of 2 and 1.33 mm, respectively.

In the presence of *Proteus* sp., the zones of inhibition showed by the different extracts were not observed to be significantly different from one another. A significantly high zone of inhibition was however observed for the control antibiotic ($p \leq 0.05$).

When tested *against Klebsiella* sp., extracts HCE and CWE recorded similar zone of inhibition of 2.67 mm,

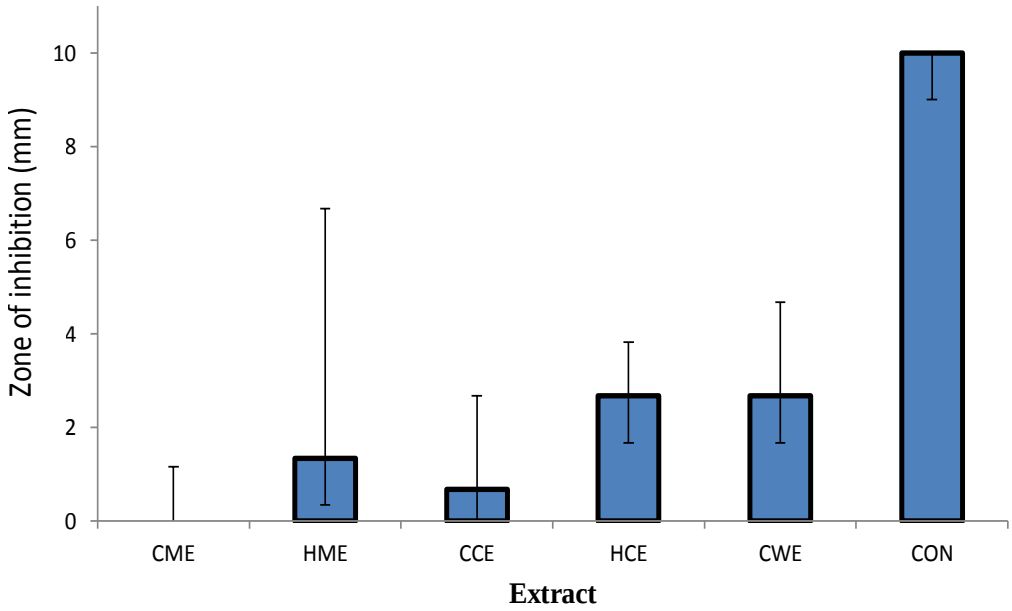

Figure 4. Antibacterial potential of the different seed extracts of the watermelon on *Klebsiella* sp.

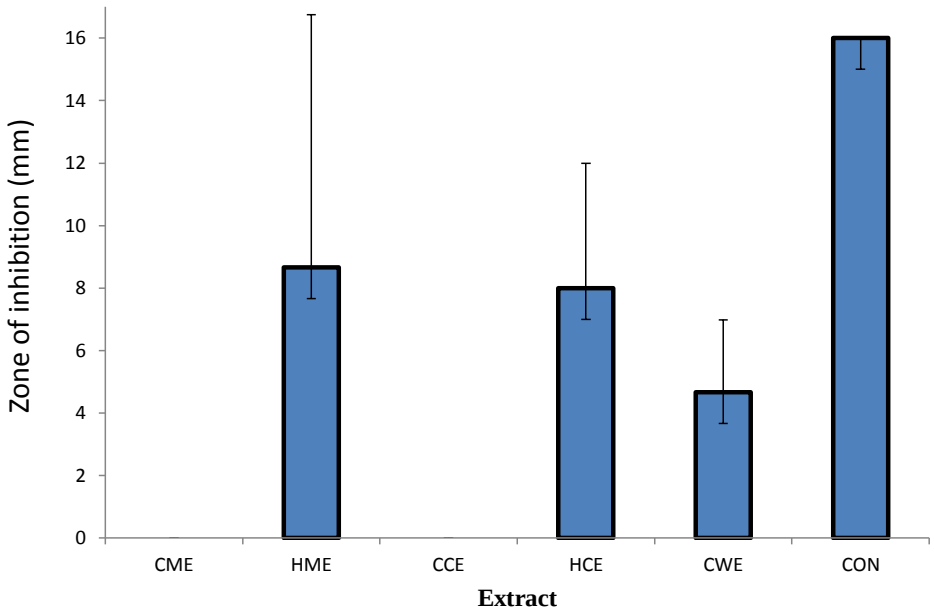

Figure 5. Antibacterial potential of the different seed extracts of the watermelon on *Pseudomonas aeruginosa.*

CME showed no zone of inhibition while the control had a zone of inhibition of 10 mm (Figure 4). As was observed in the presence of the *Proteus* sp., although the zone of inhibition observed for the control antibiotic was observed to be significantly higher than all the extracts used for the investigation, no significant differences in zones of inhibition were observed among the extracts in the presence of the *Klebsiella* sp. ($p \leq 0.05$).

In the case of *P. aeruginosa*, antibacterial activity was highest with HME (8.67 mm) followed closely by HCE (8.0 mm) while CME and CCE showed no zone of inhibition (Figure 5).

Although remarkably high zones of inhibition were observed in the presence of the *Pseudomonas aeruginosa* for the HME, HCE and CWE extracts, only the 'HME' and 'HCE' were observed to be significantly different ($p \leq 0.05$).

DISCUSSION

The presence of saponins in high concentration in CME may have resulted in the extract having the highest zone of inhibition recorded during this study. In the same vein, the concentration of saponins in HME and CWE were moderate and low respectively and this was evident in their activity against *Staphylococcus* sp. being the only Gram positive organism studied. Saponins have been reported to have antibacterial effect against Gram positive bacteria but not against Gram negative organisms (Soetan et al. 2006). The antibacterial activity of CME and HME against the *Staphylococcus* sp. in the present study can be ascribed to the presence of saponins, this was corroborated by the report of Soetan et al. (2006) and Thirunavukkarasu et al. (2010) that crude saponin extracts of *Sorghum bicolor* L. Moench and pure saponin fraction of the leaves of *Solanum trilobatum* Linn were active against *S. aureus*.

In the present study significant antibacterial effect was observed against *Staphylococcus* sp. (in the case of cold methanol and hot chloroform extracts) and *P. aeruginosa* (when the hot methanol and hot chloroform extracts were tested). This aligns with the report of Adewuyi et al. (2013) who evaluated the antibacterial activities of nonionic and anionic surfactants from *Citrullus lanatus* seed oil; they reported that *C. lanatus* seed oil did not show any inhibition against the tested organisms while the biosurfactants had activity against the growth of the test organisms, *P. aeruginosa*, *S. aureus*, *K. pneumonia* and *E. coli*. This study revealed that the *Klebsiella pneumonia* was not susceptible ($p \leq 0.05$) to any of the extracts while the *Pseudomonas aeruginosa* was susceptible to hot methanol (HME) and chloroform (HCE) extract; but Braide et al. (2012) also determined the antibacterial activity of aqueous, methanol and ethanol extracts of *C. lanatus* seed on five bacteria and found *K. pneumonia* and *P. aeruginosa* to be susceptible to all extracts. This might be as a result of the condition under which the extraction was carried out in this study; Soxhlet apparatus while that reported by Braide et al. (2012) was carried out without the introduction of any form of heat.

Conclusion

The antibacterial effects of *C. lanatus* seed extracts against the selected bacteria suggests that extracts obtained by cold maceration, Soxhlet extraction, as well as using methanol and chloroform have potential as antibacterial agents especially against *Staphylococcus* sp. and *P. aeruginosa*. Further isolation of saponins from seed and testing it against more Gram positive bacteria will be necessary to confirm that saponins are responsible for the antibacterial activity observed.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

Adewuyi A, Oderinde R, Ademisoye A (2013). Antibacterial activities of nonionic and anionic surfactants from *Citrullus lanatus* seed oil. Jundishapur J. Microbiol. 6(3):205-208.

Braide W, Odiong IJ, Oranusi S (2012). Phytochemical and antibacterial properties of the seed of watermelon (*Citrulus lanatus*). Prime J. Microbiol. Res. 2(3):99-104.

Evans WC (2002). General methods associated with the phytochemical investigation of herbal products. In: Trease and Evans Pharmacognosy (15 ed.). New Delhi: Saunders (Elsevier). pp.137-148.

Gupta A, Naraniwal M, Kothari V (2012). Modern extraction methods for preparation of bioactive plant extracts. Int. J. Appl. Nat. Sci. 1(1): 8-26.

Handa S (2008). Extraction technologies for medicinal and aromatic plants. In: Handa S, Khanuja S, Longo G and Rakesh D (Editors). United Nations Industrial Development Organization and the International Centre for Science and High Technology, Trieste, pp. 22.

Hassan LEA, Sirat HM, Yagi SMA, Koko WS, Abdelwahab SI (2011). In vitro antimicrobial activities of chloroformic, hexane and ethanolic extracts of Citrullus lanatus var. citroides (watermelon). JMPR 5(8): 1338-1344.

Meena MC, Patni V (2008). Isolation and identification of flavonoid 'quercetin' from Citrullus colocynthis (Linn.) Schrad. Asian J. Exp. Sci. 22(1):137-142.

Okunrobo OL, Uwaya OJ, Imafidon EK, Osarumwense OP, Omorodion EJ (2012). Quantitative determination, metal analysis and antiulcer evaluation of methanol seeds extract of *Citrullus lanatus* Thunb (Cucurbitaceae) in rats. Asian Pac. J. Trop. Dis. S804-S805.

Omigie IO, Agoreyo FO (2014). Effects of watermelon (Citrullus lanatus) seed on blood glucose and electrolyte parameters in diabetic wistar rats. J. Appl. Sci. Manage. 18(2):231-233.

Oseni OA, Okoye VI (2013). Studies of phytochemical and antioxidant properties of the fruit of watermelon (Citrullus lanatus). (Thunb). J. Pharm. Biomed. Sci. 27(27):508-514.

Oyeleke GO, Olagunju EO, Ojo A (2012). Functional and physiochemical properties of watermelon (*Citrullus lanatus*) seed and seed oil. IOSR J. Appl. Chem. 2(2): 29-31.

Soetan KO, Oyekunle MA, Aiyelaagbe OO, Fafunso MA (2006). Evaluation of the antimicrobial activity of saponins extract of *Sorghum biocolor* L. Moench. Afr. J. Biotechnol. 5(23): 2405-2407.

Thirunavukkarasu P, Ramanathan T, Ravichandran N, Ramkumar L (2010). Screening of Anti-microbial Effect in Watermelon (*Citrullus* sp.). J. Biol. Sci. 10:682-685.

Trease G, Evans M (1989). A Textbook of Pharmacognosy. Builler Tindall and Caussel London, 13th ed. pp. 176-180.

Varghese S, Narmadha R, Gomathi D, Kalaiselvi M, Devaki K (2013). Phytochemical screening and HPTLC finger printing analysis of *Citrullus lanatus* (Thunb.) seed. J. Acute Dis. 2(2)122-126.

Wehner TC (2008). Watermelon. In Vegetables I: Asterraceae, Brassicaceae, Chenopodicaceae and Cucurbitaceae. Springer, New York. pp. 381-418.

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Short Communication

Chemical characterization of passion fruit (*Passiflora edulis* f. *flavicarpa*) seeds

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The aim of this study was to determine the chemical characteristics of passion fruit seeds and their oil for possible use in human food and reduction of organic waste from fruit industrialization. Passion fruit seeds were analyzed for moisture, lipids, proteins, ash, fibers, titratable acidity, pH, soluble solids and antioxidant activity. The oil was characterized for parameters such as acid value, saponification, iodine and peroxide. The content of oil extracted demonstrates that it has good potential for industrial utilization. According to analyses, the oil has characteristics similar to conventional edible oils such as soybean, and may be a new source of human consumption. Passion fruit seeds have high nutritional value, proving to be a promising product, mainly because it contains significant amounts of proteinase. Therefore, passion fruit seeds and their oil should be used as raw material in the food, chemical and pharmaceutical industry, as they have beneficial features.

Key words: Industrial utilization, characterization, by-products.

INTRODUCTION

Brazil stands out as the world's largest producer of passion fruit (*Passiflora edulis flavicarpa*), and produced in 2010 approximately 920,000 tones of the fruit (IBGE, 2012), and there is a worldwide trend toward consumption of passion fruit due to its great nutritional value, that is, the consumption of exotic tropical fruits with distinctive flavor is increasing.

Passion fruit is native to tropical America and widely grown in Brazil. It is rich in vitamin C, calcium and phosphorus. The most economically importance of passion fruit is in the form of concentrated juice (Ferrari et al., 2004); however, for industrialization, passion fruit bark and seeds are usually discarded, and these represent over 60% of the fruit and are almost always treated as organic waste.

An alternative to by-products from passion fruit industrialization would be its use in human food. The seeds of these fruits are rich in fiber, minerals and lipids, with good amount of proteins (Oliveira et al., 2011). Chau and Huang (2004) reported that passion fruit seeds are

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rich in crude lipids and insoluble dietary fiber. Passion fruit seed oil has pleasant taste and mild odor and compares with the cottonseed oil in nutritional value and digestibility (Ferrari et al., 2004). Passion fruit seeds contain large amounts of oil and fiber and are generally removed after being crushed. These residues imply in operating costs for industries and may become an environmental problem. Thus, the extraction of oil from passion fruit seeds can add value to this agroindustrial residue (Malacrida and Jorge, 2012).

Passion fruit seed oil has physicochemical characteristics similar to some common edible oils; may be a new source for human consumption (Kobori and Jorge, 2005). Passion fruit seed oil has high contents of polyunsaturated fatty acids that can be and successfully used, for example, in the production of margarine, which are consumed without heat treatment and therefore less susceptible to oxidation (Lopes et al., 2010).

Many substances present in fruits, pulp, seeds and bark can contribute to beneficial effects such as antioxidant activity (Zeraik et al., 2010). The action of antioxidants present in plant extracts plays an important role in reducing lipid oxidation in plant and animal tissues, because when incorporated in human food not only preserves the quality of food, but also helps reduce the risk of the development of diseases such as arteriosclerosis and cancer (Namiki, 1990; Ramarathnam et al., 1995).

Passion fruit seed oil, extracted by Soxhlet, has significant antioxidant quantity and can serve as a source of natural antioxidants preventing the development of diseases or as a food additive, increasing the stability and quality of food products (Malacrida and Jorge, 2012). The aim of this study was to determine the chemical characteristics of passion fruit seeds and their oil for possible use in human food and reduction of organic waste from fruit industrialization.

MATERIALS AND METHODS

Passion fruit seeds were obtained in a farm in the municipality of Santa Helena de Goiás, GO, with an altitude of 525 m and coordinates 17° 37' 58"S and 50° 33' 20" W.

Passion fruits were sent to the Laboratory of Fruits and Vegetables, Food Engineering Unit, Federal Institute of Education, Science and Technology of Goiás, Rio Verde Campus, Goiás, where seeds were extracted. Subsequently, the wet samples were dried in the sun for 6 hours to remove the residual moisture from fruits.

The physical and chemical composition of fresh pequis was determined as follows: moisture according to methodology No. 925.09 of AOAC (2000), until constant weight was achieved; ether extract according to methodology No. 925.38 of AOAC (2000); crude protein content according to micro-Kjeldahl method No. 920.87 of AOAC (2000); ash, according to the gravimetric method of AOAC (2000) No. 923.03, and calcined at 550°C, with permanence of the sample inside the FORNITEC oven, model 1926, Brazil; crude fiber according to methodology of AOAC (1995). The titratable acidity contents were obtained by titration of the filtered juice with NaOH solution (0.01 N), and the results were expressed as % citric acid (IAL, 2005). The content of soluble solids, expressed in °Brix, was determined by reading the filtered juice in refractometer model Atago N-2E (IAL, 2005). The pH was determined using a Bel Engineering pH meter; model W3B, according to the technique of IAL (2005).

Table 1. Chemical and nutritional propertied and antioxidant activity of passion fruit seeds.

Component	Value*
Soluble solids (°Brix)	3.50 ± 0.27
pH	6.36 ± 0.08
Titratable acidity (%)	0.90 ± 0.05
Proteins (%)	11.80 ± 0.20
Lipids (%)	30.22 ± 1.42
Moisture (%)	7.45 ± 0.16
Ash (%)	2.05 ± 0.35
Dietary fiber (%)	67.23 ± 1.69
Antioxidant activity (EC_{50})	108 ± 1.58

*****Mean and standard deviation.

The acid, peroxide, iodine and saponification values were determined by adapted official methodology described by “Institute Adolfo Lutz” (IAL, 2008).

The measure of the scavenging activity of DPPH free radical was performed according to methodology described by Brand-Williams and Berset (1995). Passion fruit seed samples were ground (10 g) extracted into tubes with methanol (40 ml) and centrifuged for 5 min; the supernatant was stored for further use. The extracts were diluted in three different concentrations for subsequent spectrophotometric reading.

The reaction mixture consisted of adding 0.5 ml sample, 3 ml of absolute ethanol and 0.3 ml of DPPH free radical solution and 0.3 mM of ethanol. The reduction of the DPPH free radical was measured by reading the absorbance at 517 nm in 100 min of reaction. The antioxidant activity was expressed according to Equation 1 of Mensor et al. (2001) described below:

$$\% AA = 100 - \{[(Ab_{ssample} - Abs_{blank}) \times 100] / Abs_{control}\} \quad (1)$$

Where, Aa = sample absorbance; Ab = blank absorbance; Ac = control absorbance. Thus, the specific blank of extracts in various concentrations used for each sample was taken into account. The specific sample blank was determined using 3.3 ml of ethanol and 0.5 ml of sample in each concentration and the absorbance was read at 517 nm after 100 min of reaction. A tube containing 3 ml of absolute ethanol, 0.5 ml of 70% ethanol and 0.3 ml of 0.5 mM DPPH served as a negative control.

RESULTS AND DISCUSSION

The results for the passion fruit seeds studied are shown in Tables 1 and 2. Soluble solids are composed of water-soluble compounds, including sugars (Chitarra and Chitarra, 2005). The passion fruit seeds used in this study had average soluble solids content of 3.5 °Brix.

The moisture content of seeds was 7.45%. The moisture reported in this study was higher than the value found by Jorge et al. (2009) of 6.89%; however, is within

Table 2. Chemical characteristics of the passion fruit seed oil.

Component	Value*
Acidity value (mg KOH g^{-1})	1.63 ± 0.08
Iodine index (g I/100 g oil)	109.48 ± 5.02
Peroxide index (meq/Kg)	1.54 ± 0.12
Saponification Index (mg KOH g^{-1})	173.95 ± 1.48

*****Mean and standard deviation.

the recommended limit for plant flours established by law, which is 15% (ANVISA , 2005), which ensures higher quality, because the drier the flour, the higher its microbiological stability.

The titratable acidity is consistent with expectations, and passion fruit seeds have low acidity and pH near neutral, with 0.90% and 6.36, respectively.

The amount of lipids was found to be 30.22%, which indicates that passion fruit seeds are good source of oils, especially when compared to soybean seeds, which contains according to Costa et al. (2005), about 20% in oil. Togashi et al. (2007) used passion fruit seeds in the diets for broilers and found about 24.5% of lipid content. Passion fruit seed oil has high contents of unsaturated fatty acids, which indicates that this product has good potential for use in both human and animal feed, as in the cosmetics industry (Ferrari et al., 2004).

Passion fruit seeds have high amounts of dietary fiber, equal to 67.23%. Fiber intake is associated with lower concentrations of cholesterol, lower risk of coronary heart disease, reduced blood pressure, increased weight control, improved glycemic control, reduced risk of certain forms of cancer, and improved gastrointestinal function (Anderson et al., 1994).

The high fiber and protein content of passion fruit seeds suggests that these can be used as animal and human feed due to their significant nutritional value since as long as there are no toxic or allergenic substances (George, 2009).

The antioxidants present in passion fruit seed extracts react with DPPH, which is a stable radical and converts it into 2,2- diphenyl - 1 - picryl hydrazine, where the degree of discoloration indicates the antioxidant potential of the extract (Roesler, 2007). Plants, particularly fruits, are rich in several compounds with antioxidant activity, which include ascorbic acid, polyphenols and carotenoids (Melo et al., 2008). The EC_{50} for passion fruit extract was 108 $\mu g.mL^{-1}$. Roesler (2007) studied the antioxidant activity of fruits from the cerrado regions and obtained results similar to those of this study in relation to the seeds of fruits studied, obtaining 162.97 $\mu g.mL^{-1}$ for lobeira pulp + seed and 30.97 $\mu g.mL^{-1}$ for araticum seeds. Since passion fruit seeds are often treated as industrial waste, its antioxidant activity reveals that this seed can and should be considered extremely significant material for consumption, as the antioxidant activity is related to the reduction of diseases. The study of bioactive substances is of great importance and these studies reveal the importance of the consumption of plant products due to their high antioxidant activity.

The chemical characteristics of passion fruit seed oil are shown in Table 2. The acidity index provides important data on the condition of the oil conservation. The Codex Alimentarium Commission (2008) determines the maximum value of the acidity index of 4.0 mg $KOHg^{-1}$ as a quality parameter. The value found in this study (1.63 mg KOH g^{-1}) indicates that the studied oil can be used for food purposes, since it lies within the stipulated quality values.

The iodine index is related to the amount of unsaturation present in the oil. Passion fruit seed oil is mostly composed of unsaturated fatty acids (Ferrari, 2004), with iodine value of 109.48 g/2/100 g of oil. Industrially, this parameter is used as a way to control the hydrogenation of oils. Malacrida and Jorge (2012) found in their work iodine index for passion fruit seed oil of 128.0 gI2/100 g of oil. With iodine index above 100, the oil can be considered as semi-drying (Bello et al., 2011).

The determination of the peroxide value is used as an indicator of lipid oxidation. The Codex Alimentarium Commission (2008) establishes for refined and crude oils maximum peroxide values between 10 and 15 meq O_2kg^{-1}. The oil under study showed 1.54 $meqO_2$ kg^{-1} peroxide value. Ferrari et al. (2004) found higher peroxide value working with passion fruit seeds, of 4.7 meq O_2 kg^{-1}. High peroxide values indicate that, somehow, the oil was exposed to oxidative process either during the preparation of the raw material, extraction or oil storage (Jorge and Luzia, 2012).

The saponification index indicates the mean molecular weight of fatty acids esterified to glycerol in the triacylglycerol molecule; a high saponification index indicates fatty acid of lower molecular weights and vice-versa (Jorge and Luzia, 2012). The value found for the saponification index of the sample analyzed was 173.95 mg $KOHg^{-1}$, which is compatible with values found by Kobori and Jorge (2005) when working with tomato, orange, passion fruit and guava oil samples of 172.86, 181.05, 174.97 and 189.91 mg KOH g^{-1} oil, respectively.

Conclusion

The disposal of industrialization products is a problem and adding value to these products, in this case passion fruit seeds, have a great economical, scientific or technological importance.

The content of oil extracted demonstrates that it has good potential for industrial utilization. According to the analyses, it has features similar to conventional edible oils such as soybean oil, and may be a new source for human consumption.

Passion fruit seeds have high nutritional value, proving

to be a promising product, mainly due to their significant amounts of protein and fiber. The use of seeds and their oil should be encouraged, as they provide the development of clean technology, reducing organic waste and increasing food production for containing high nutritional values.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA (ANVISA) (2005). Resolução RDC n° 263, de 22 de setembro de 2005. Regulamento técnico para produtos de cereais, amidos, farinhas e farelos. Disponível em: <http://www.suvisa.rn.gov.br/contentproducao/aplicacao/sesap_suvisa/arquivos/gerados/resol_263_set_2005.pdf>. Acess in: 30 outubro 2014.

Anderson JW, Smith BM, Gustafson NJ (1994). Health benefits and practical aspects of high-fiber diets. Am. J. Clin. Nutr. 59(5): 1242S-1247S.

Bello MO, Akindele TL, Adeoye DO, Oladimeji AO (2011). Physicochemical Properties and Fatty Acids Profile of Seed Oil of *Telfairia occidentalis* Hook, F. Int. J. Basic Appl. Sci. 11(6):9-14.

Chau CF, huang YL (2004). Characterization of passion fruit seed fibres - a potential fibre source. Food Chem. 85:189-194.

Codex Alimentarius Commission (2008). Codex-Stan 210: Codex standard for named vegetable oils. Rome.

Costa NP, Mesquita CM, Maurina AC, Neto JBF, Krzyanowski FC, Oliveira MCN, Henning AA (2005). Perfil dos aspectos físicos, fisiológicos e químicos de sementes de soja produzidas em seis regiões do Brasil. Rev. Bras. Sementes 27:2.

Ferrari RA, Colussi F, Ayub RA (2004). Caracterização de subprodutos da industrialização do maracujá - Aproveitamento das Sementes. Rev. Bras. Frutic. 26:101-102.

Instituto Brasileiro de Geografia e Estatística (IBGE) (2012). Produção Agrícola Municipal – PAM. Banco de Dados SIDRA. Disponível em: http://www.ibge.gov.br. Acesso em 11 de dezembro de 2013.

Jorge N, Luzia, DMM (2012). Caracterização do óleo das sementes de Pachira aquatica Aublet para aproveitamento alimentar. Acta Amaz. 42:1.

Jorge N, Malacridra CR, Angelo PM, Andreao D (2009). Composição centesimal e atividade antioxidante do extrato de sementes de maracujá (*passiflora edulis*) em óleo de soja. Pesqui. Agropecu. Trop. 39:380-385.

Kobori CN, Jorge N (2005). Caracterização dos óleos de algumas sementes de frutas como aproveitamento de resíduos industriais. Ciênc. Agrotec. 29:1008-1014.

Lopes RM, Sevilha AC, Faleiro FG, Silva DB, Vieira RF, Agostini-Costa TS (2010). Estudo comparativo do perfil de ácidos graxos em semente de Passifloras nativas do cerrado brasileiro. Rev. Bras. Frutic. 32:2.

Malacrida CR, Jorge N (2012). Yellow passion fruit seed oil (Passiflora edulis f. flavicarpa): physical and chemical characteristics. Braz. Arch. Biol. Technol. 55(1):127-134.

Melo EA, Maciel MIS, Lima VLAG, Nascimento RJ (2008). Capacidade antioxidante de frutas. Rev. Bras. Ciênc. Farm. 44:2.

Namiki M (1990). Antioxidants/antimutagens in food. Crit. Rev. Food Sci. Nutr. 29:273-300.

Oliveira SEM, Regis AS, Resende ED (2011). Caracterização dos resíduos da polpa do maracujá-amarelo. Cienc. Rural 41:4.

Ramarathnam, N, Osawa, T, Ochi, H, Kawakishi, S (1995). The contribution of plant food antioxidants to humans health. Trends Food Sci. Nutr. 6:75-82.

Roesler R, Malta LG, Carrasco LC, Holanda RB, Sousa CAS, Pastore GM (2007). Atividade antioxidante de frutas do cerrado. Ciênc. Tecnol. Alimentos 27:53-60.

Togashi CK, Fonseca JB, Soares RTRN, Gaspar A, Detmann E (2007). Composição em ácidos graxos dos tecidos de frangos de corte alimentados com subprodutos de maracujá. Rev. Bras. Zootec. 36(6): 2063-2068.

Zeraik M, Pereira L, CAM Zuin VG, Yariwake JH (2010). Maracujá: um alimento funcional?. Rev. Bras. Farmacogn. 20(3):459-471.

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Full Length Research Paper

Mycosynthesis of iron nanoparticles by *Alternaria alternata* and its antibacterial activity

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Nanotechnology is one of the most emerging fields in the recent years. In the current investigation, we report the biosynthesis of iron nanoparticles (Fe-NPs) employing *Alternaria alternata* fungus, which is an eco-friendly process for the synthesis of metallic nanoparticles. Fe-NPs were synthesized through the reduction of aqueous Fe^{3+} ion in the dark conditions. Ultraviolet–visible spectrum of the aqueous medium containing iron ion showed a peak at 238 nm and another peak at 265 nm. The forming of nanoparticles was confirmed by transmission electron microscope, scanning electron microscope and energy-dispersive x-ray. The morphology of nanoparticles is found to be cubic shapes mostly and the average particle diameter as determined by scanning electron microscope was found to be 9±3 nm. Fe-NPs showed antibacterial activity against both Gram-positive and Gram-negative bacteria used in this study due to its oxidative damage for bacterial cell wall. Iron nanoparticles show more antimicrobial activity to *Bacillus subtilis* than *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Key words: Mycosynthesis, *Alternaria alternata*, iron nanoparticles, antibacterial.

INTRODUCTION

Nanobiotechnology is defined as the study of biological phenomenon at nanosize scale. Generally, Nanotechnology comprises the study of materials that are less than 100 nm in size. Recently, the study of nanosize particles has gained much attention due to their unique size-dependent properties and their various applications (Habeeb, 2013). The advantages in nanotechnology make us to apply the concepts in a variety of fields. The unique characteristics of nanomaterials over their macroscaled counter-parts gave them high importance in a lot of valuable applications due to their altered physical and chemical properties (Feynman, 1991). In addition to their special physical and chemical characteristics, they showed unusual optical, photoelectrochemical and electronic properties (Peto et al., 2002). The problems with the physical and chemical methods used for the production of nanoparticles such as: short time stability and safety issues can be solved by the use of other biosynthetic methods such as the use of microorganisms. Many organisms can produce either extracellular or

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intracellular inorganic substances (Mann, 1996). Magnetite nanoparticles are examples of nanoparticles produced by unicellular magnetotactic bacteria (Lovley et al., 1987). The use of fungi in biosynthesis of nanoparticles was discovered recently. Fungi are eukaryotes which are characterized by production of large amounts of enzymes. They are relatively more complicated than prokaryotes in terms of genetic manipulation when used in over expression of enzymes included in nanoparticles biosynthesis. Ultraviolet-visible spectra analysis of biologically synthesized iron nanoparticles in a culture media of *Pleurotus* sp. fungus that grow in $2x10^{-4}$ M $FeSO_4$ solutions for 72 h showed formation of nanoparticles at 226 and 276 nm wavelength. Transmission electron microscope images reflect depositions of particles in both inside as well as outside indicating the biosynthesis process. X-Ray fluorescence of control samples do not shows any iron elements but was present in treated mycelium (Mazumdar and Haloi, 2011). Gold nanoparticles were formed by *Verticillium* fungus that was exposed to 10^{-4} M $HAuCl_4$ solution which was indicated by appearance of purple colour in the fungus biomass (Mukherjee et al., 2001a).

The mechanism of gold nanoparticles biosynthesis by *Verticillium* sp. includes the electrostatic interaction between ions and enzymes carboxylate groups at first, followed by reduction of ions by the enzymes which are present in the cell wall of the fungus mycelia resulting in nuclei formation which grow and accumulate more and more forming these nanoparticles (Mukherjee et al., 2001b). The emergence of infectious diseases in general poses a serious threat to public health worldwide, especially with the emergence of antibiotic-resistant bacterial strains. Generally, both Gram-positive and Gram-negative bacterial strains are thought to present a major public health problem. Over the years, antibiotics have been used to control infections resulting from both community and hospital environments (Lowy, 1998; Komolafe, 2003; Hawkey, 2008). *Staphylococcus aureu*s is one of the most common human pathogens, and leads to many types of infection (Grinholc et al., 2008). *S. aureus* is also known to possess an increasing ability to resist antibiotics (such as penicillin, methicillin, tetracycline, erythromycin, and vancomycin) (Jevons, 1961; Hiramatsu, 2001).

Thus, it is necessary to find an alternative treatment (perhaps without the use of antibiotics) for bacterial infection and bacterial pollution. Iron nanoparticles are used recently over a wide range in different applications such as the use of metallic nanoparticles especially, as potential antimicrobials (Tran et al., 2010). This antimicrobial property of iron nanoparticles can be used in many applications such as in water treatment, food processing, textiles industry and, construction, medicine and food (Vasilache et al., 2011). Advances in nanotechnology are producing an accelerated proliferation of new nanomaterial composites that are likely to become an important source of engineered health-related products. Nanoparticles with antifungal effects are of great interest in the formulation of microbicidal materials (Nuñez-Anita et al., 2014). Metallic nanoparticles with antibacterial properties represent a promising alternative approach to antibiotics. Their complex mechanism of action influences different bacterial structures and gives them advantages compared to antibiotics with more specific mechanism of action. Targets of nanoparticles are outer and inner bacterial structures cell wall, plasma membrane, proteins, and DNA (Kon and Rai, 2013).

In this study we report the biologically synthesized iron nanoparticles by *A. alternata* fungus. The antibacterial activity of iron NPs were tested against Gram-positive and Gram-negative bacteria.

MATERIALS AND METHODS

Fungus

A. alternata (RCMB-009002) used for the synthesis of Fe-NPs, was procured from The Regional Centre for Mycology and Biotechnology, Al-Azhar University, Egypt.

Growth culture and synthesis of Fe-NPs

Fungal biomass of *A. alternata* used for biosynthetic experiments was grown in liquid medium containing (g/l): KH_2PO_4 7.0, K_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.1, $(NH_4)_2SO_4$ 1.0, yeast extract 0.6, glucose 10.0. Erlenmeyer flasks were inoculated with spores and incubated at 28ºC with shaking (150 rpm) for 72 h. After the incubation, the biomass was filtered (Whatman filter paper No. 1) and then extensively washed with distilled water to remove any medium component. Fresh and clean biomass was taken into Erlenmeyer flasks containing 100 ml of Milli-Q water. The flasks were agitated at the same conditions as described above, then the biomass was filtered again and cell-free filtrate was used in experiments. Two hundred and fifty (250) millilitre of iron (III) nitrate (1 mM of final concentration) was mixed with 1 g of harvested and washed mycelia of *A. alternata* was suspended and plugged with cotton then incubated at 28°C in dark with shaking (150 rpm) for 72 h. Control (without fungus) was also run along with the experimental flasks. After incubation time fungal mycelia was removed by centrifugation at 4,000 rpm for 10 min and the resulting supernatant was used for further analytical studies for extracellular Fe-NPs (Mazumdar and Haloi, 2011).

Characterization of Fe-NPs

The preliminary detection of Fe-NPs was carried out by visual observation of colour change of supernatant. These samples were later subjected to optical measurements, which were carried out by using a UV-visible spectrophotometer (Unicam-UV2 UV/Vis Spectrometer) and scanning the spectra between 200 and 800 nm at the resolution of 1 nm. A scanning electron microscope (SEM) (JEOL JSM-5500LV) was used to characterize the structure properties of the synthesized iron nanoparticles. The element composition of the synthesized materials was identified by energy dispersive X ray microanalysis system (EDX) (Module Oxford 6587 INCA x-sigh) coupled to the SEM at 17 KV after gold coating using SPI-Module sputter coater. The morphology and particles size of

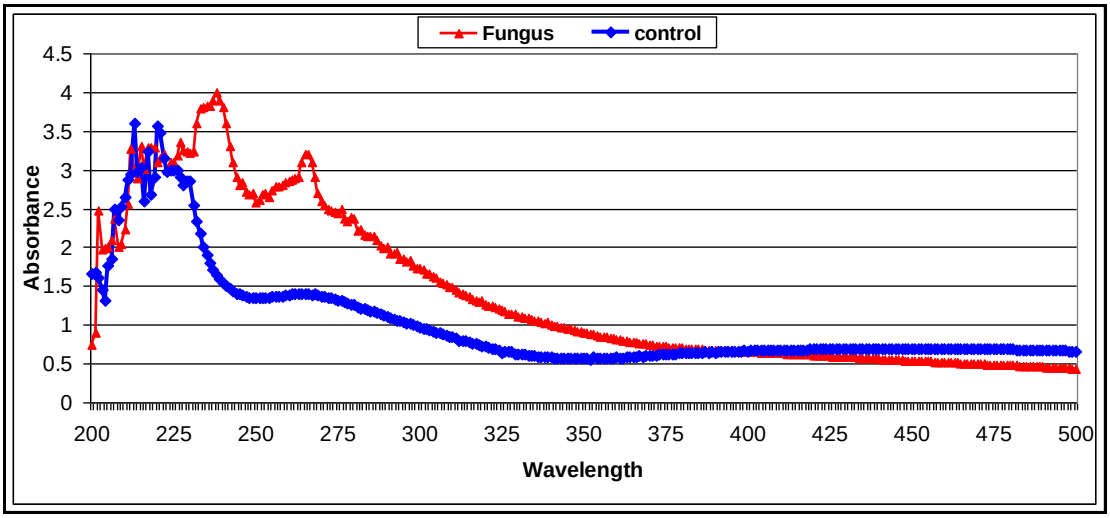

Figure 1. UV-Vis spectra of iron nanoparticles biologically synthesized by fungus.

the resulting nanoparticles were analyzed using transmission electron microscope (TEM) (Jeol JEM 1200 EXII) connected to a high resolution imaging system. Samples for TEM studies were prepared by placing drops of the iron nanoparticles solutions on carbon-coated TEM copper grids.

Determination of antibacterial activity of Fe-NPs by well-diffusion method

Antimicrobial activities of the synthesized iron nanoparticles were performed against both Gram-negative (*Escherichia coli* RCMB 0100052 and *Pseudomonas aeruginosa* RCMB 0100043) and Gram-positive (*Bacillus subtilis* RCMB 010067 and *Staphylococcus aureus* RCMB 0100028) bacteria. The antibacterial activity was done by modified Kirby-Bauer well diffusion method (Azam et al., 2012). In brief, the pure cultures of organisms were subcultured in Müller-Hinton broth at 35±2°C on a rotary shaker at 160 rpm. For bacterial growth, a lawn of culture was prepared by spreading the 100 µL fresh culture having 10^6 colony-forming units (CFU)/mL of each test organism on nutrient agar plates with the help of a sterile glass-rod spreader. Plates were left standing for 10 min to let the culture get absorbed. Then 6 mm wells were punched into the nutrient agar plates for testing nanomaterial antimicrobial activity. Wells were sealed with one drop of molten agar (0.8% agar) to prevent leakage of nanomaterials from the bottom of the wells. Using a micropipette, 100 µL Fe-Nps suspension was poured onto each well on all plates. After overnight incubation at 35±2°C, the different levels of zone of inhibition were measured. Antibiotics Gentamicin and Ampicillin were used as a positive control for Gram-negative and Gram-positive bacteria respectively.

TEM observations of treated *Bacillus subtilis*

Bacterial cells of both treated and untreated samples were observed under TEM (JEOL 1010, Japan). The samples after centrifugation and washing were fixed in 25% glutaraldehyde/ paraformaldehyde in cacodilate buffer at room temperature for 1 h. Then samples were fixed in 1% osmium tetraoxide. Sample embedding was carried out using a standard protocol (Croft, 1999) and 60 nm thick slices were cut with a diamond knife (LBR ultratome III). The slices were deposited on bare 200 mesh copper grids, and stained with 2 wt% uranyl acetate for 5 min. Finally, the grids were dried in a desiccator and examined using TEM, for study biocidal action of nanoparticles and any morphological changes, and then photos were taken by special digital camera (Canon, Japan).

RESULTS AND DISCUSSION

The biosynthesis of iron nanoparticles was carried out by exposure of a precursor salt aqueous iron (III) nitrate solution of 1 mM concentration of fungal cell-free filtrate obtained by incubating the fungus *A. alternata* (RCMB-009002) in an aqueous solution. The reaction was carried out at 28°C in dark with shaking (150 rpm) for 72 h. The mycelium growth was found to be slow and there was a gradual change in colour of medium as well as the mycelium. Oxidation process of ferric (Fe^{3+}) ions may be takes place in culture solutions. Iron nanoparticle was green synthesized by fungus *Aspergillus oryzae* TFR9 using $FeCl_3$ as a precursor metal salt (Tarafdar and Raliya, 2013). The efficacy of silver synthesized biolarvicide with the help of entomopathogenic fungus, *Beauveria bassiana*, was assessed against the different larval instars of dengue vector, *Aedes aegypti* (Banu and Balasubramanian, 2014).

The absorption in the visible range directly affects the perceived colour of the chemicals involved. In this region of the electromagnetic spectrum, molecules undergo electronic transitions. The UV-visible spectrum of Fe-NPs in fungus media supernatant is shown in Figure 1. The two absorption peaks at wavelengths of 238 and 265 nm indicate the formation of iron nanoparticles. Iron oxide nanoparticle shows the peak at 222 nm (Pal, 2014). The analysis of iron nanoparticles that forming by *Pleurotus* sp. under UV-vis spectrophotometer showed nearly peaks at wavelengths 226 and 276 nm (Mazumdar and

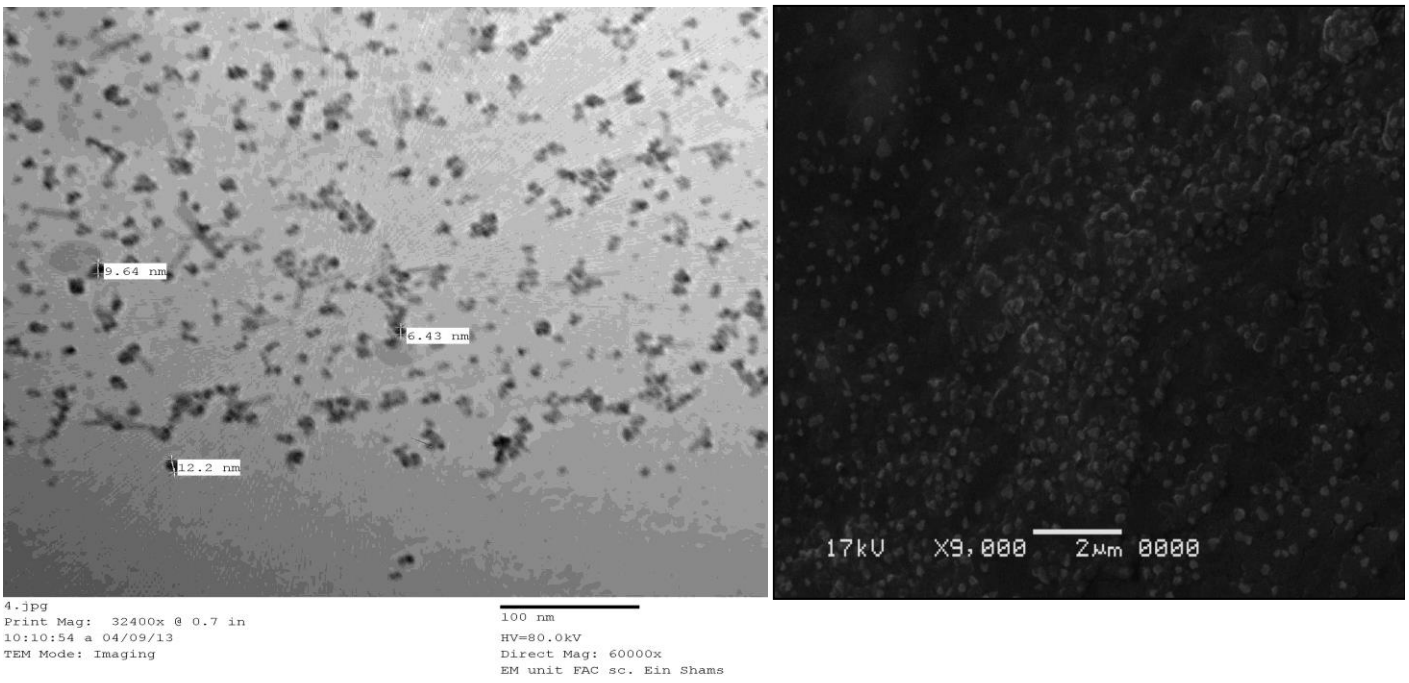

Figure 2. TEM and SEM images for synthesized iron nanoparticles.

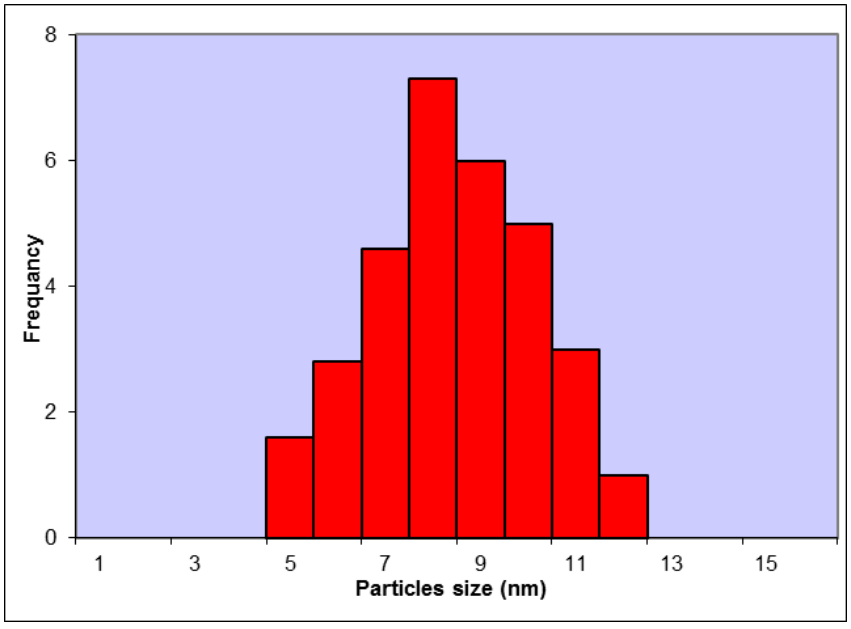

Figure 3. Particles size distribution of synthesized iron nanoparticles.

Haloi, 2011). On the other hand, two absorption peaks of Fe-NPs that synthesized by *Sargassum muticum* aqueous extract were introduced at wavelengths 402 nm and 415 nm (Mahdavi et al., 2013) this shifts in peaks of nanoparticles may be due to size of particles.

The TEM and SEM images for produced Fe-NPs showed smaller particles in form of cubic shapes, while the average particle diameter as determined by TEM was found to be 9±3 nm, where particles size ranged from 5.4 to 12.1 nm (Figures 2 and 3). However, the mean diameter average size of iron oxide nanoparticles synthesized by bioreduction of ferric chloride solution with

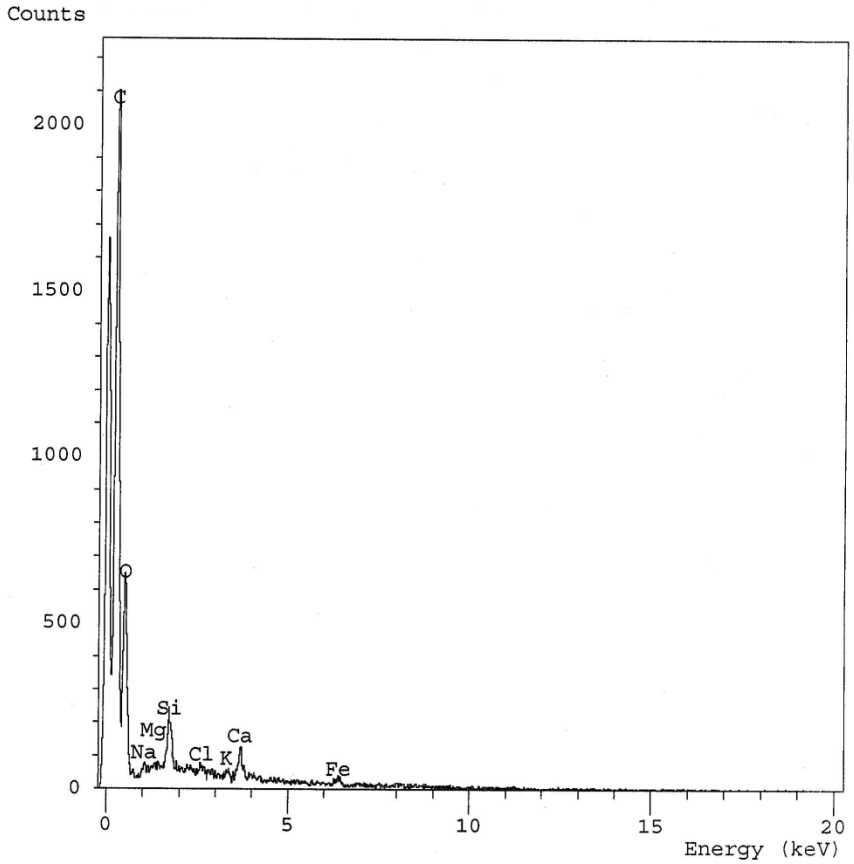

Figure 4. Spectrum of iron nanoparticles obtained by EDX microanalysis.

a green method using brown seaweed (*Sargassum muticum*) aqueous extract contain sulphated polysaccharides as the reducing agent was 18±4 nm and cubic shapes (Mahdavi et al., 2013). Moreover, iron oxide nanoparticles biologically synthesized using an aqueous extract of *Passiflora tripartita mollissima* fruit showed average particle size of spherical 22.3±3 nm (Kumar, et al., 2014).

In the EDX spectra pattern of filtered fungus growth media (Figure 4), the peaks around 0.1, 1.8, and 4.9 kV are related to the binding energies of Fe. Therefore, the pattern showed diffraction narrow peaks in the figure confirm the crystalline structure of iron nanoparticles without any impurity peaks. However, the results of XRD analysis for the synthesized nanoparticles that were obtained with different initial concentrations of $FeCl_3$ indicated that the particles consist of γ-Fe_2O_3 and α-Fe_2O_3. It also revealed that a concentration of 0.02 M of $FeCl_3$ resulted in the particles with the smallest Z-average diameter (Cheng et al., 2012).

The mechanism of Fe-Nps formation by fungi was discussed by some authors (Figure 5), AuNPs formation can occur either in the intracellular or extracellular space. Extracellular AuNPs formation is commonly reported for fungi when Au^{3+} ions are trapped and reduced by proteins in the cell wall. Previous work with the fungus *Verticillium* sp. ruled out the possibility that reduced sugars in the cell wall are responsible for the reduction of Au^{3+} ions and suggested adsorption of $AuCl^{4-}$ ions on the cell-wall enzymes by electrostatic interaction with positively charged groups (for example, lysine) (Mukherjee et al., 2002; Duran et al., 2011; Das et al., 2012).

Antibacterial activity results revealed that iron nanoparticles (synthesized from iron (III) nitrate 1 mM) acted as excellent antibacterial agents against both Gram-positive and Gram-negative bacteria. Table 1 and Figures 6 and 7 show the zone of inhibition produced by iron nanoparticles against both Gram-positive and Gram-negative bacterial strains. Fe-NPs exhibited maximum (16.4 mm) bacterial growth inhibition against *B. subtilis*, in the form of zone-of-inhibition studies, where diffusion of nanoparticles on nutrient agar plates inhibits growth. In contrast, Fe-NPs showed zones of inhibition of 13.2, 12.3 and 10.5 mm, respectively, against *E. coli, S. aureus* and *P. aeruginosa.* In the presence of iron oxide nanoparticles growth of both *B. subtilis* and *E. coli* strain is inhibited. Iron oxide nanoparticles show more antimicrobial activity to *B. subtilis* than *E. coli* (Pal, 2014). Moreover, iron oxide nanoparticles have excellent

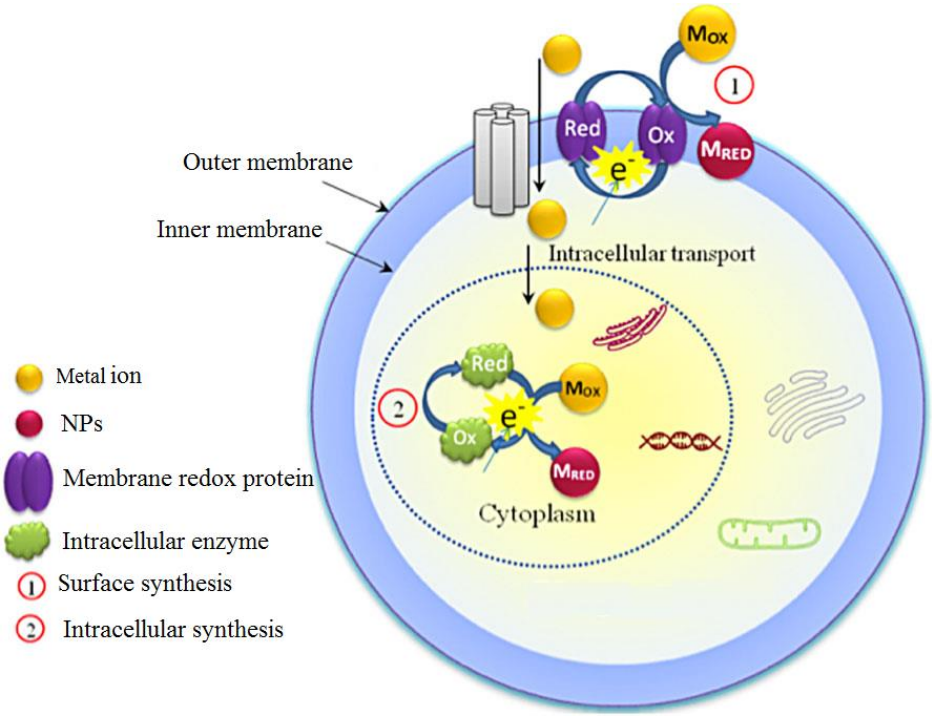

Figure 5. Schematic diagram of a proposed mechanism of metal nanoparticles biosynthesis in cell fungus (Das et al., 2012).

Table 1. Inhibition zones of iron nanoparticles compared with antibiotics.

Bacterial species	Inhibition zone (mm)		
	Fe-NPs	Ampicillin	Gentamicin
Bacillus Subtilis	16.4±0.7	27.4±0.2	-
Staphylococcus aureus	12.3±0.5	32.4±0.1	-
Escherichia coli	13.2±0.6	-	22.3±0.2
Pseudomonas aeruginosa	10.5±0.3	-	17.3±0.1

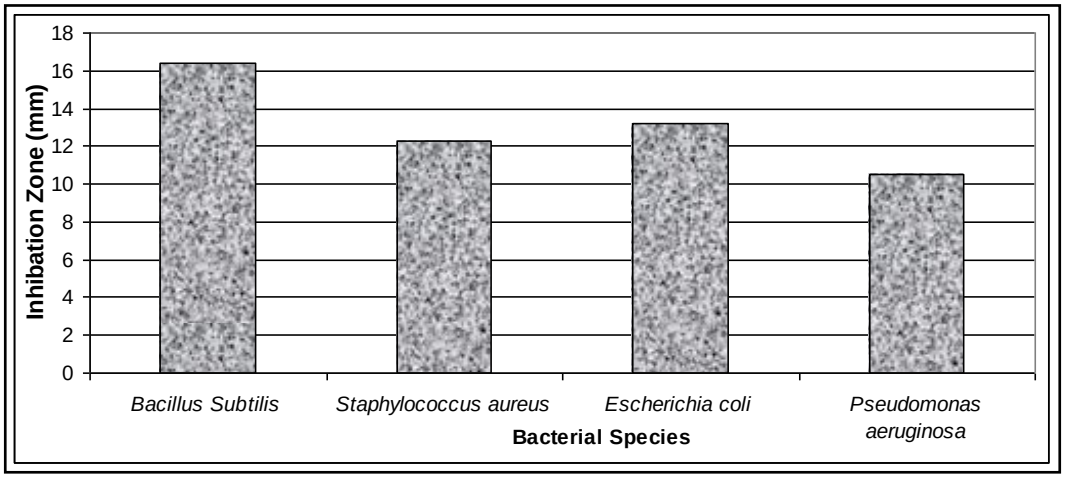

Figure 6. Inhibition zones of Fe-NPs for treated Gram-positive and Gram-negative bacteria.

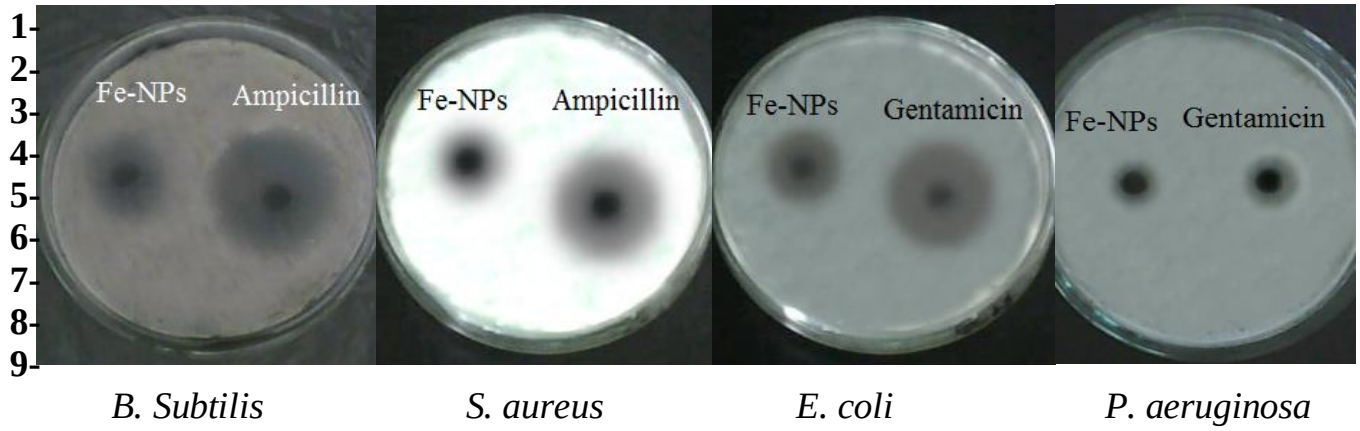

Figure 7. Inhibition zones of Fe-NPs against treated bacterial species.

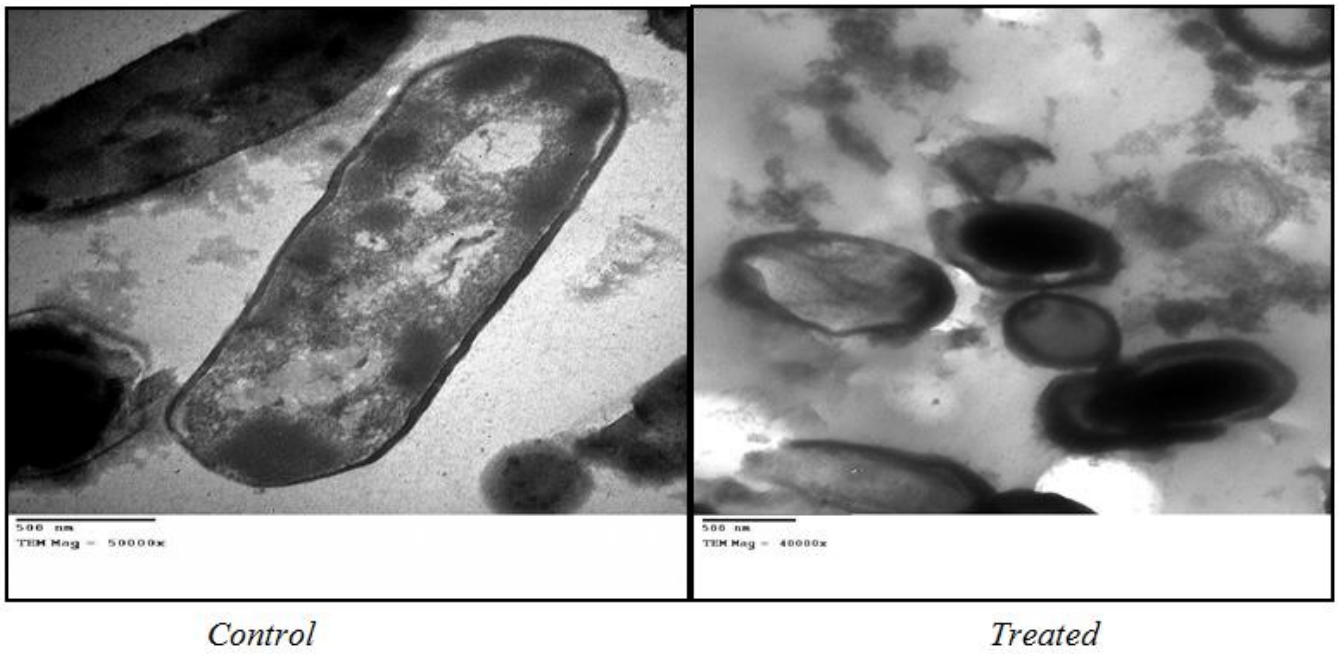

Figure 8. TEM micrographs of treated and untreated *Bacillus subtilis* bacteria with Fe-NPs.

antibacterial activity against *S. aureus* and promising Sono-Fenton catalytic ability for dye discoloration (Harifia and Montazer, 2014). The antibacterial activity of ZnO NPs was 72, 80, 88 and 84% more effective than Fe_2O_3 NPs, while 28, 31, 27, 50 and 40% more bactericidal than CuO NPs against *E. coli*, *S. aureus*, *P. aeruginosa*, and *B. subtilis*, respectively (Azam et al., 2012).

Transmission electron microscopy showed morphological changes of *B. subtilis* bacterial cells (Figure 8), Fe-NPs might cause oxidative stress via reactive oxygen species generation and the Fenton reaction. Oxidative stress in *B. subtilis* can result from disturbance of the electronic and/or ionic transport chains due to the strong affinity of the nanoparticles for the cell membrane. Similar results of zero-valent iron NPs toxicity on *E. coli* have been detected, where iron, as a strong reductant, might induce the decomposition of functional groups in membrane proteins and lipopolysaccharides, or Fe-NPs could be oxidized by intracellular oxygen, leading to oxidative damage via the fenton reaction. When Fe-NPs penetrate cells through disrupted membranes, it causes further physical damage and death (Lee et al., 2008).

Conclusions

Study report the biosynthesis of iron nanoparticles (Fe-NPs) employing *A. alternate* fungus, which is an eco-

friendly process for the synthesis of iron nanoparticles. Fe-NPs were synthesized through the reduction of aqueous iron (III) nitrate solution of 1 mM concentration in the dark conditions. The forming of nanoparticles was confirmed by UV-visible spectrum, TEM, SEM and EDX. The morphology of nanoparticles is found to be cubic shapes mostly and the mean size was 9±3 nm. Fe-NPs showed antibacterial activity against both Gram-positive and Gram-negative bacteria, but iron nanoparticles show more antimicrobial activity to *B. subtilis* than *E. coli, S. aureus* and *P. aeruginosa*.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

Azam A, Ahmed AS, Oves M, Khan MS, Habib SS, Memic A (2012). Antimicrobial activity of metal oxide nanoparticles against Gram-positive and Gram-negative bacteria: a comparative study. Int. J. Nanomed. 7:6003–6009.

Banu N, Balasubramanian C (2014). Myco-synthesis of silver nanoparticles using *Beauveria bassiana* against dengue vector, *Aedes aegypti* (Diptera: *Culicidae*). Parasitol. Res. 113(8): 2869-2877.

Cheng Z, Kuan Tan AL, Tao Y, Shan D, Ting KE, Yin XJ (2012). Synthesis and characterization of iron oxide nanoparticles and applications in the removal of heavy metals from industrial wastewater. Int. J. Photoenergy 2012, Article ID 608298:1-5

Croft S (1999). Methods in molecular biology: electron microscopy methods and protocols. p. 117.

Das SK, Liang J, Schmidt M, Laffir F, Marsili E. (2012) Biomineralization mechanism of gold by zygomycete fungi Rhizopous oryzae. ACS Nano 6:6165-6173.

Duran N, Marcato PD, Duran M, Yadav A, Gade A, Rai M (2011). Mechanistic aspects in the biogenic synthesis of extracellular metal nanoparticles by peptides, bacteria, fungi, and plants. Appl. Microbiol. Biotechnol. 90:1609-1624.

Feynman R (1991). There's plenty of room at the bottom. Science 254:1300-1301.

Grinholc M, Szramka B, Kurlenda J, Graczyk A, Bielawski KP (2008). Bactericidal effect of photodynamic inactivation against methicillin resistant and methicillin susceptible *Staphylococcus aureus* is strain-dependent. J. Photochem. Photobiol. A Chem. 90(1):57-63.

Habeeb MK (2013). Biosynthesis of nanoparticles by microorganisms and their applications. Int. J. Adv. Sci. Technol. Res. 1(3):44-51.

Harifia T, Montazer M (2014). *In situ* synthesis of iron oxide nanoparticles on polyester fabric utilizing color, magnetic, antibacterial and sono-Fenton catalytic properties. J. Mater. Chem. B 2:272-282.

Hawkey PM (2008). The growing burden of antimicrobial resistance. J. Antimicrob. Chemother. 62 Suppl. 1: i1-i9.

Hiramatsu K (2001). Vancomycin-resistant *Staphylococcus aureus*: A new model of antibiotic resistance. Lancet Infect Dis. 1(3):147-155.

Jevons MP (1961). Celbenin"-resistant Staphylococci. BMJ 1(5219):124-125.

Komolafe OO (2003). Antibiotic resistance in bacteria – an emerging public health problem. Malawi Med. J. 15:63-67.

Kon K, Rai M (2013). Metallic nanoparticles: mechanism of antibacterial action and influencing factors. J. Comp. Clin. Pathol. Res. 2(1):160-174.

Kumar B, Smita K, Cumbal L, Debut A (2014). Biogenic synthesis of iron oxide nanoparticles for 2-arylbenzimidazole fabrication. J. Saud. Chem. Soc. 18(4): 364–369.

Lee C, Kim JY, Lee WI, Nelson KL, Yoon J, Sedlak DL (2008). Bactericidal effect of zero-valent iron nanoparticles on *Escherichia coli*. Environ. Sci. Technol. 42:4927-4933.

Lovley DR, Stolz JF, Nord GL, Philips EJP (1987). Anaerobic production of magnetite by a dissimilatory iron-reducing microorganism. Nature 330:252-254.

Lowy F (1998). *Staphylococcus aureus* infections. N. Engl. J. Med. 339:520-532.

Mahdavi M, Namvar F, Ahmad MB, Mohamad R (2013). Green biosynthesis and characterization of magnetic iron oxide (Fe_3O_4) nanoparticles using seaweed (*Sargassum muticum*) aqueous extract. Molecules 18: 5954-5964.

Mann S (1996). Biomimetic materials chemistry. VCH, New York: 1-40.

Mazumdar H, Haloi N (2011). A study on biosynthesis of iron nanoparticles by *Pleurotus* sp. J. Microbiol. Biotechnol. Res. 1(3):39-49.

Mukherjee P, Ahmad A, Mandal D, Senapati S, Sainkar SR, Khan MI, Ramani R, Parischa R, Ajayakumar PV, Alam M, Sastry M, Kumar R (2001a). Bioreduction of $AuCl_4^-$ ions by the fungus *Verticillium* sp. and surface trapping of the gold nanoparticles formed. Angew. Chem. Int. Ed 40:3585-3588.

Mukherjee P, Ahmad A, Mandal D, Senapati S, Sainkar SR, Khan MI, Ramani R, Ajayakumar PV, Alam M, Kumar R, Sastry M (2001b). Fungus mediated synthesis of silver nanoparticles and their immobilization in the mycelial matrix: a novel biological approach to nanoparticle synthesis. Nano Lett. 1:515-519.

Mukherjee P, Senapati S, Mandal D, Ahmad A, Khan MI, Kumar R, Sastry M (2002). Extracellular synthesis of gold nanoparticles by the fungus *Fusarium oxysporum*. Chem. Biochem 3:461-463.

Nuñez-Anita RE, Acosta-Torres LS, Vilar-Pineda J, Espinosa JCM, Hernandez JF, Castanõ VM (2014). Toxicology of antimicrobial nanoparticles for prosthetic devices. Int. J. Nanomed. 9: 3999-4006

Pal S (2014). Antimicrobial activity of iron oxide nanoparticles. M.Sci. Thesis, National Institute of Technology Rourkela-769008, Orissa, India.

Peto G, Molnar GL, Paszti Z, Geszti O, Beck A, Guczi L (2002). Electronic structure of gold nanoparticles deposited on SiOx/Si (100). Mater. Sci. Eng. C 19:95-99.

Tarafdar JC, Raliya R (2013). Rapid, low-cost, and ecofriendly approach for iron nanoparticle synthesis using *Aspergillus oryzae* TFR9. Journal of Nanoparticles Volume 2013, Article ID 141274:1-4. http://dx.doi.org/10.1155/2013/141274

Tran N, Mir A, Mallik D, Sinha A, Nayar S, Webster TJ (2010). Bactericidal effect of iron oxide nanoparticles on *Staphylococcus aureus*. Int. J. Nanomed. 5:277-283.

Vasilache V, Popa C, Filote C, Cretu MA, Benta M (2011). Nanoparticles applications for improving the food safety and food processing. 7th International Conference on Materials Science and Engineering – BRAMAT Braşov, February: 24-26.

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Full Length Research Paper

Physicochemical parameters and antibiotics residuals in Algerian honey

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The aim of the present study was to evaluate the quality of 36 samples of different honey type supplied by local producers from Algeria in order to verify its compliance with the standards of Codex Alimentarius and European Union (EU). For that, five physicochemical parameters were analyzed using the HPLC method: hydroxyl-methyl furfural (HMF), sugars, diastase activity and search of antibiotic contamination with streptomycin and tetracycline. The physicochemical analyses of the Algerian honeys show that 56% of samples correspond to Codex standards and 44% not in conformity with the standards required by the Codex Alimentarius and EU, because part of the samples had one or more defects. The percentage not in conformity was due to the high rates of hydroxyl-methyl furfural, sucrose and also to the low enzyme level. Analysis performed by the laboratory to detect residues of tetracycline and streptomycin in honey have revealed insignificant traces of oxytetracycline in two samples of honey (0.03 ppb). From the present study, it is observed that the Algerian honey samples is not completely in agreement with the requirements of international honey standards which could be caused by inappropriate actions during processing and storage steps.

Key words: Honey quality, sugar, diastase activity, hydroxymethylfurfural, antibiotic residues.

INTRODUCTION

The Codex Alimentarius (2001) define honey as a natural sweet substance, produced by honeybees from the nectar of plants or from secretions of living parts of plants, or excretions of plant-sucking insects on the living

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parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature. Honey composition basically depends on the nectar composition of each producing plant species, conferring specific characteristics to it (Marchini et al., 2007). Honey is a complex mixture which presents very great variations in composition and characteristics due to its geographical and botanical origin, its main features depend on the floral origin or the nectar foraged by bees (Kebede et al., 2012; Saxena et al., 2010).

The composition and quality of honey also depend on several environmental factors during production such as weather and humidity inside the hive, nectar conditions and treatment of honey during extraction and storage. However, quality and composition of honey are negatively affected by other factors such as overfeeding with sucrose and other sucrose variants, harvesting prior to maturity, unhealthy storage conditions and overused veterinary drugs (Sahinler et al., 2004).

Honey is composed primarily of the sugar: glucose and fructose; its third greatest component is water (Singh et al., 2012). Honey also is composed of a complex mixture of carbohydrates and other less frequent substances, such as organic acids, amino acids, proteins, minerals, vitamins, lipids (Blasa et al., 2006; Ball, 2007; Zerrouk et al., 2011), aromatic compounds, flavonoids, vitamins, pigments, waxes, pollen grains, several enzymes and other phytochemicals (Gomes et al., 2010; Lazarevic et al., 2010; de Almeida-Muradian et al., 2013).

According to the Codex Alimentarius and Council Directive of the European Union (EU), honey is a natural product and should be exempt of contaminant. On the other hand, contamination of honey may occur through the common use of antibiotics such as the streptomycin and its derivative dihydrostreptomycin (DHSTR) which often combined with tetracycline. These antibiotics are used as veterinary drugs or crop-protection agents in broad-spectrum anti infection formulation (Michel et al. 2004) because they are against both Gram-positive and few Gram-negative bacteria (Kwapong et al., 2013).

In order to guaranties the nomination of honey and also protect human health, the use of antimicrobials in apiculture is usually strictly regulated or banned. According to Regulation (EC) No 470/2009 and Regulation (EU) No 37/2010, in the European Union, no maximum residue level (MRL) for tetracycline and any other antibacterial substance residues in honey are allowed (Cara et al., 2012).

Considering the nutritional properties of honey and its scarcity in the Algerian market, it is exposed to fraud. To check its quality, various international institutions, such as the International Honey Commission (IHC), the Codex Alimentarius and the European Commission propose methods of analyses to ensure that honey is authentic in respect to the legislative requirements.

Algerian honey production is estimated to average 33,000 quintals in 2011 with a yield of 4-8 Kg/hive (Oudjet, 2012), which is less than the needs of local consumption while it is supposed to be at the origin of an important export outlet. This low production affects the price and makes it remain high. Therefore, consumption remains as low as production. This lack of production is the result of multiple causes such as absence of national regulation, lack of a professional organization and insufficient quality control laboratory (Bendeddouche and Dahmani, 2011). Nevertheless, Algerians researchers and scientists try to establish correct denominations to assure a minimum marketing level of the product.

The aim of the present study was to evaluate the quality of 36 samples belonging to different honey type supplied from local producers from Algeria in order to verify its compliance with the standards of Codex Alimentarius, 2001 and the Council of the European Union (EU), 2002. Five physicochemical parameters were analysed using the HPLC method: hydroxyl-methyl furfural, sugars, diastase activity and search of antibiotic contamination with streptomycin and tetracycline.

MATERIALS AND METHODS

Sampling

Thirty six (36) honey samples produced in various regions of Algeria (Figure 1) were collected directly from beekeepers or from apicultural corporations or from beekeepers vendors of trade fairs (Local open air markets) between July and September 2012. All honey samples were labeled either according to their botanical and geographical origin as suggested by the beekeepers; sampling was accompanied by an interview with the beekeeper for information on these honeys as date of harvest and mode of extraction, or according to the testimonies of the vendors. The samples were stored in a refrigerator at 4-6°C in airtight plastic containers until analysis.

Technical analysis

Determination of diastase activity

The diastase activity was measured using the Phadebas amylase test tablets purchased from (Megazyme) (Phadebas method), insoluble blue-dyed, cross-linked starch was used as the substrate for the degradation reaction (Sak-Bosnar and Sakac, 2012), according to the International Honey Commission (Bogdanov, 2002). This is hydrolysed by the enzyme, yielding blue water-soluble fragments, determined photometrically at 620 nm with molecular Devices Spectramax 340 Microplate Reader. The absorbance of the solution is directly proportional to the diastase activity of the sample. The diastase activity, expressed as DN or diastase number, was calculated from the absorbance measurements using Equations (1) and (2), respectively:

$$DN = (28.2 \cdot \Delta A_{620}) + 2.64 \quad (1)$$

$$DN = (35.2 \cdot \Delta A_{620}) - 0.46 \quad (2)$$

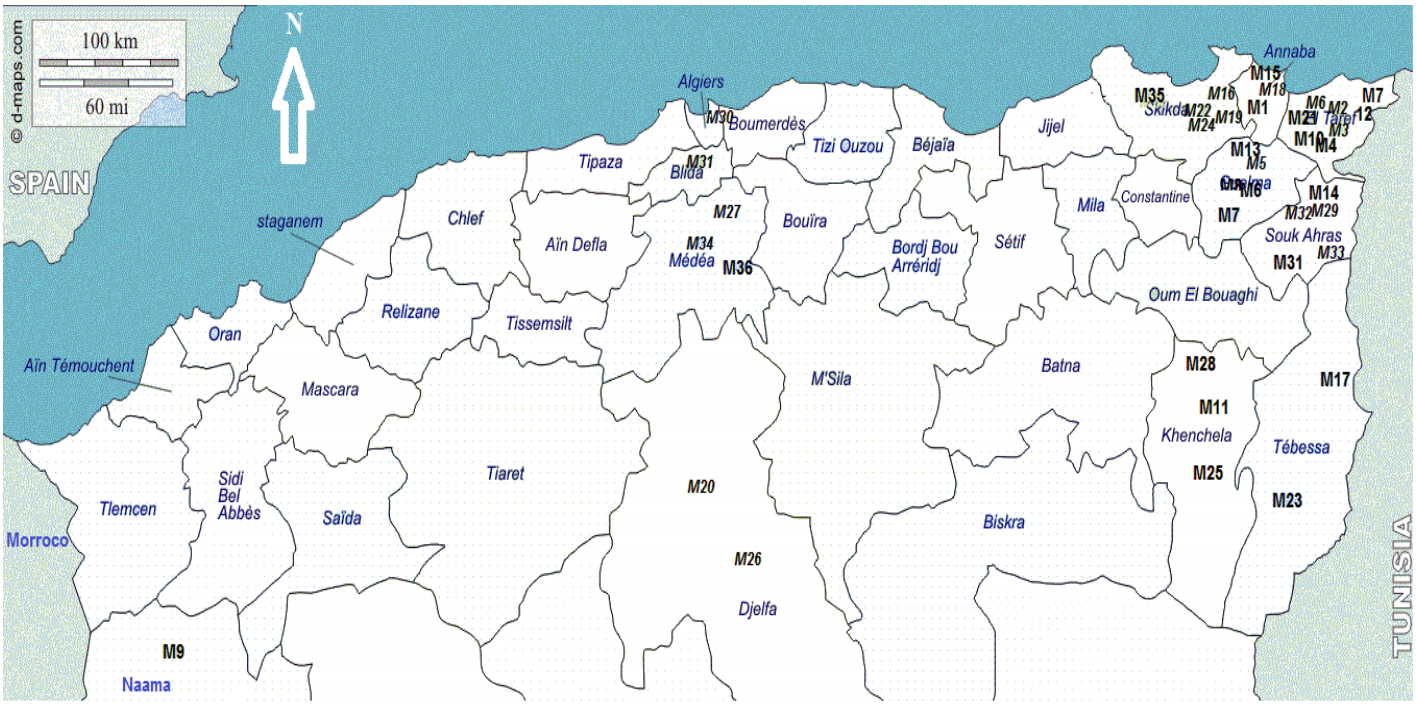


Figure 1. Geographical origins of Algerian honey samples.

For either high (8 to 40 diastase units) or low (up to 8 diastase units), respectively, Equations 1 and 2 are suggested by the International Honey Commission (Bogdanov, 2002). Diastase activity was referred to as diastase number (DN) in the Schade scale, which corresponds to the Gothe scale number, or to gram of starch hydrolysed per hour at 40ºC per 100 g of honey.

Determination of sugars

Determination of sugars in honey was established in the current study by the use of a chromatographic system (HPLC SHIMADZU LC-20 AD) with RI detector (IOP) and Column chromatography apHera NH_2 150 mm x 4.60 mm x 5 microns (or equivalent). Fructose, glucose and sucrose were measured, according to the analytical methods harmonized by the European Honey Commission (Bogdanov, 2002).

The chemical reagents used for HPLC analysis were acetonitrile and methanol (HPLC grade) from Sigma- Aldrich (Milan, Italy). The sugar standards used were fructose, glucose, sucrose of 99.5% purity from Sigma- Aldrich (Milan, Italy).

10.00 ± 0.01 g of honey was weighed in a beaker of 100 ml and dissolved with distilled water (40 ml) which was quantitatively transferred in graduated flask. After that, 25 ml of methanol was added to the solution and completed to 100 ml with distilled water. The solution was filtered with a nylon filter (0.45 µm), and the first 2 ml was discarded and further chromatographic analysis under the following operating conditions was done: Flow rate of 1.5 ml / min, injection volume of 20 µl, and detector temperature of 30 ± 1°C. The acquisition time was 10 min. Identification of the sugars was carried out by comparing the retention times of the peaks of the sample solution with that of the reference solution. The sugar concentration was calculated by comparing the peak area of the sample corresponding to the peak of the reference solution.

Determination of HMF

The method determines the concentration of 5-(hydroxymethyl-) furan-2-carbaldehyde (HMF) (Fallico et al., 2004). The determination of HMF was carried out in solutions of honey samples (5.00 ± 0.01 g of honey) and diluted to 50 ml with distilled water, filtered on 0.45 µm filter and injected into an HPLC (HPLC SHIMADZU LC-20 AD) equipped with: pump UV detector, auto sampler, column thermostating system, data acquisition and processing system. The HPLC was chromatography on reverse phase column with dimensions of 150 x 3 mm, packed with octadecylsilane (C18), containing particles of a diameter of 2.7 microns. The HPLC conditions were the following: isocratic elution with 85% water and 15% methyl alcohol. All the solvents were of HPLC grade (Sigma-Aldrich). The column was thermostated at 34°C; at flow rate of 1.0 ml / min and injection volume of 4 µl. The wavelength range was 285 nm, and acquisition time was 3 min. HMF was identified from the peak in honey with a standard HMF from Sigma-Aldrich (Milan, Italy), and by comparison of the spectra of the HMF standard with that of one honey samples. The amount of HMF was determined using an external calibration curve, measuring the signal at λ=285 nm.

Analysis of residues

The quantitative analysis of residues of streptomycin was performed with HPLC technique according to Albino et al. (2005). Sample (5 g) was mixed with 20 ml of extracting solution (sodium heptasulphonate (0.05 M) and sodium hydrogen phosphate (0.08 M, pH=2). The solution was then vortexed for 30 min and centrifuged for 10 min. Streptomycin was eluted with methanol and evaporated by a Rota vapor (60°C, 250 mbar). The residues were recovered with 1 ml of sodium dodecylsulfate (70 mM). The mixture

was vortexed and then put in ultrasonic bath for 5 min (Gallina et al., 2005; Baggio et al., 2009). Streptomycin was detected and quantified by the external standard calibration. HPLC analyses were performed, using post-column derivatization, performed in 1 ml of reaction coil placed in a column heater (55°C), and fluorimetric detection system (SHIMADZU 20 AD HPLC) with fluorescence detection (SHIMADZU, Japan) and chromatographic column Alltech (Alltech, Italia) C18 Platinum 5 µM 250 mm x 4.60 mm. After filtration, a 100 ml aliquot of residue solution was injected into the chromatographic system. The streptomycin was analyzed at 1 ml /min of flow with an isocratic elution of mobile phase 40% of sodium dodecyl sulfate (0.1 M) + sodium 1,2-naphthoquinone-4-sulfonate (0.5 mM) and 60% of acetonitrile for 15 min. Post-column, a sodium hydroxide solution 0.2 M was added (0.4 ml /min) in flow. Excitation and emission wavelengths of 263 and 435 nm, respectively, were used to detect the streptomycin. Samples quantification was performed using the external standards. This approach allows determining the streptomycin in honey in the range of concentrations ranging from 5 to 200 ng/kg. The positive results were confirmed by co-chromatographic method according to Decision 657/2002/CE.

This method allows the determination of tetracyclines (tetracycline, oxytetracycline, chlortetracycline and doxycycline) in honey in the range of concentrations ranging from 3 to 30 ng/g. A honey sample (5 g) was dissolved with 25 ml extraction buffer (succinic acid 0.1 M, pH 4). The sample solution was vortexed and then centrifuged for 10 min at 4000 *g*. The supernatant layer was recovered and purged in a metal-chelating affinity column (MCAC) cartridge. Tetracyclines were eluted with McIlvaine EDTA solution (citric acid, disodium hydrogen phosphate, EDTA and sodium chloride). The MCAC cartridge was prepared by fulfilling with chelating sepharose fast-flow resin (1.5 ml) and conditioned with copper sulfide solution (0.01 M), the quantitative analysis of any residues of tetracycline was performed with HPLC-MS technique according to Gallina et al. (2005), Cristofani et al. (2009) and Baggio et al. (2009) with some modification, using HPLC SHIMADZU coupled to LCMS-2010 EV rivelatore and chromatographic column C18MS (100 mm x 2,1) 5 µm X TERRA (Waters). The flow rate was 0.6 ml/min; Injection volume was 20 µl, at room temperature with a gradient for elution of mobile phase consisting of 0.5% formic acid + methanol +acetonitrile (50/50). The wavelength used to detect the TCs was 365 nm. The positive results were confirmed by co-chromatographic method according to Decision 657/2002/CE.

The standard solutions were obtained by Sigma-Aldrich. The solvents used were HPLC grade (99.9%) and the other chemicals were all of analytical grade from Sigma-Aldrich (Milan, Italy). Before being applied for HPLC analyses, all solutions were filtered by micro-filter (4.5 µm).

Statistical analyses

Physic-chemical results were compared with International Regulatory Standards. Means, standard deviations and the correlation coefficient (HMF-Diastase index) were calculated by using the software (Statistica version 10).

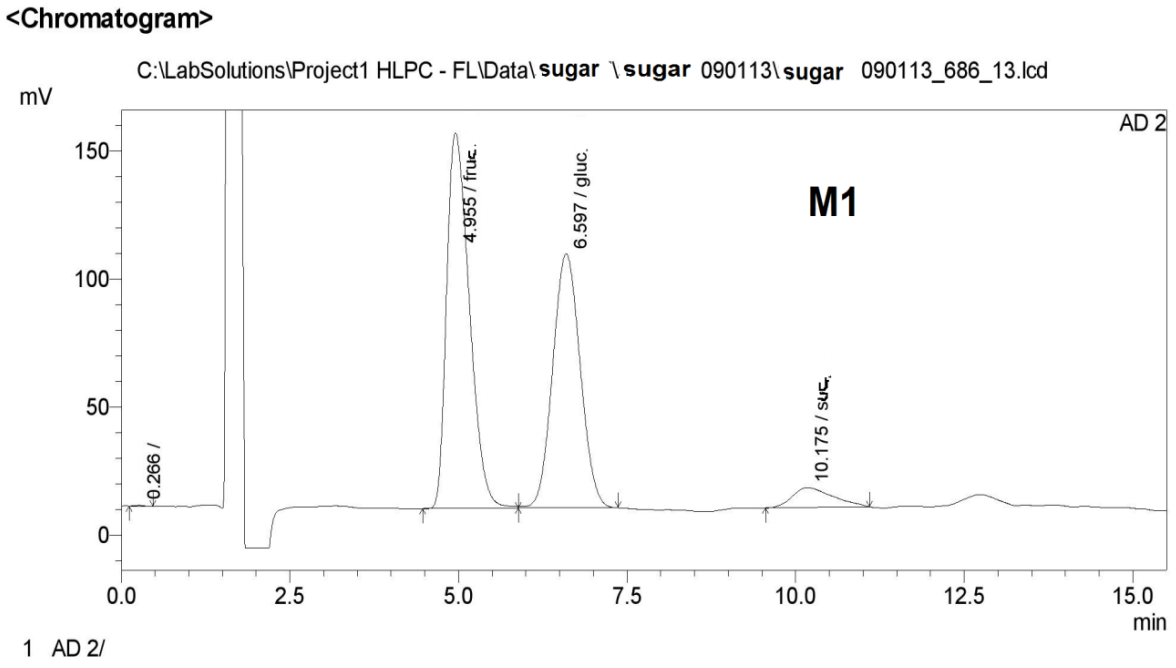
RESULTS AND DISCUSSION

The physicochemical analyses carried out on Algerian honey showed that 56% of samples met Codex standards and 44% did not, because a part of them have one or more defects (Table 1). The latter was due to the high rates of hydroxyl-methyl furfural, sucrose and also to the low enzyme level.

The HMF for the 36 honey samples analyzed in the present work ranged from 0.170 to 571.9 mg/kg and revealed that 26 of them (72%) had levels below the limits of HMF Codex acceptable standard (≤40 mg/kg) according to Codex Alimentarius (2001) and Council Directive of EU (2002), indicating the use of good practices by beekeepers. Eleven (11%) had a rate of HMF between 40 and 80 mg/kg and showed inadequate HMF content. According to White (1992), honey samples from subtropical countries may have naturally high HMF values regardless of the fact that the honey was not overheated or adulterated, due to high temperatures (Silva et al., 2013). However, nine samples were considered of unacceptable quality with very high values of HMF. These values relate to samples M5, M19, M27, M29, M34, and M36 and can be explained by inadequate treatment of these honeys probably overheating (Singh and Bath, 1998; Kubis and Ingr, 1998; Zappalà et al., 2005; Zerrouk et al., 2011), poor storage conditions and old honey (Khalil et al., 2010). Besides that, it is reported that extremely high >500 mg/kg HMF values demonstrate an adulteration with invert syrup (Coco et al., 1996; Popa et al., 2009; Ajlouni and Sujirapinyokul, 2010), also it is reported that the use of high fructose corn syrup as sweetener can lead to high HMF content reaching 100-1000 mg/kg (Makawi et al., 2009). Thus, there is a very high possibility that the honeys bought from the open-air markets are adulterated, since their HMF values are higher than 500 mg/kg.

These studies have yielded similar results to previous results (Bendeddouche and Dahmani, 2011; Zerrouk et al., 2013; Ouchemoukh et al., 2007). Jilani et al. (2008) reported for Tunisian multifloral honeys HMF values which also showed heat influence (HMF ranged between 3.0-39.6 mg/kg). In Morroco, Chakir et al. (2011) have obtained values of HMF limited between 0.09 and 53.38 mg/kg, but four honey samples contained a high HMF value which included between 90.76 and 783 mg/kg. The hot Algerian climate may also be the origin of this phenomenon as similar heat damage was also observed in the mean diastase number (Makhloufi et al., 2010). However, it is essential to quantify this component in order to check on product quality (Marchini et al., 2007), because HMF is a compound that may be mutagenic (Sommer et al., 2003; Glatt et al., 2005). Furthermore, it may also be carcinogenic (Kowalski et al., 2013) and cytotoxic (Islam et al., 2014).

Diastase is a natural enzyme of honey. The diastase activity measured the combined activities of both α-amylases and β-amylases which were secreted from bee salivary (Vit and Pulcini, 1996; Chua et al., 2014). Enzyme activity in honey depends on the intensity of the



Quantitative Results

detector

ID#	Name	Ret. Time	Area	Height	Conc.
1	Fructose (Fruc)	4.955	3433078	146574	0.000
2	Glucose (Gluc)	6.597	2815284	99243	0.000
3	Sucrose(Suc)	10.175	324092	7793	0.000

Figure 2. Sugar chromatograms of some Algerian honey (example of sample M 1).

nectar flow and the amount of nectar the bee processes in each period (Escuredo et al., 2011). In addition, the diastase activity and the diastase content varies according to floral source (White et al., 1962). Diastase and invertase activities are commonly used in Europe as an indicator for honey freshness (Manzanares et al., 2011). This is because the enzyme activities decrease in heated or old honey.

Nine samples (M5, M6, M16, M19, M21, M27, M29, M34 and M36) have a lower diastase index than the minimum standard value (superior than 8) from 00 - 45.30; the scale with an average value of 17.58 ± 12.27. The result can be due to either, overheating by beekeeper, or to the natural poor levels of amylase in the sample because the diastase activities in honey vary in wide limits depending on botanical origin of honey (Persano-Oddo et al., 1990) and thus, have a limited freshness indicating power; HMF is regarded as better quality criterion in this respect (Buba et al., 2013). Both HMF and diastase activity are the international parameters used to control the limit for thermal treatment to honey (Chua and Adnan, 2014). The correlation test between HMF content and diastase activity showed strong negative correlation (r = -0.605436). This confirms that, those two parameters are inversely proportionate to each other.

Glucose and fructose are the main sugars in honey and their actual proportion depends largely on the source of the nectar (Anklam, 1998). The sugars of honey were determined by HPLC and an example of chromatogram is presented in Figure 2. The results of the sugar analysis of all the 36 honey samples (Table 1) show that the fructose contents varied between 29.33 and 42.39% with an average of 37.61 ± 3.11%. The glucose contents of the samples were within a range of 25.38 -37.65% with a mean value of 31.88 ± 3.39%. In our study, 85.5% of the honey samples analyzed had fructose as the dominating sugar. In respect to reducing sugars (fructose and glucose), the EC Directive 2001/110 imposes reducing sugars ≥60 g/100 g, except for honeydew honey, which is ≥45 g/100 g. The reducing sugar contents varied between 60.18 and 79.29 g/100 g with an average of 69.50± 4.40 g/100 g. Our results met this standard and are similar to other published levels for reducing sugars. Furthermore, the reducing sugar content of the honey tested was similar with the findings of other previously

Table 1. Results of physicochemical analysis of 36 samples (Mean values ± standard deviations).

Sample	ID (Gothe scale)	HMF (ppm)	Sucrose (%)	Fructose (%)	Glucose (%)	Reducing sugar (%)	G/F ratio
M1	17.1±0.32	12.4±0.54	3.4±0.15	38.72±0.53	32.47±0.25	71.49±0.007	1.19±0.006
M2	20.81±0.02	5.6±0.04	2.09±0.09	42.00±0.16	27.15±0.27	69.18±0.02	1.54±0.01
M3	25.57 ±0.54	13.9±0.15	2.99±0.01	38.79±0.24	32.82±0.28	71.61±0.01	1.18±0.001
M4	12.95±0.32	30.9±0.97	2.50±0.61	38.78±0.28	32.34±0.96	71.11±0.02	1.20±0.02
M5	0.091±0.009	571.9±0.94	5.91±0.05	34.17±0.27	36.89±0.16	71.08±0.02	0.92±0.002
M6	6.46±0.06	73.5±0.1	1.20±0.08	40.43±0.44	34.60±0.13	75.02±0.02	1.16±0.004
M7	27.50±0.39	10.4±0.39	2.71±0.11	38.07±0.38	32.20±0.31	70.29±0.01	1.18±0.01
M8	31.25±0.35	9.7±0.25	2.47±0.02	38.63±0.009	32.36±0.08	70.97±0.04	1.19±0.005
M9	24.6±0.1	5.1±0.05	2.72±0.18	37.14±0.37	33.88±3.23	72.07±3.92	1.1±0.09
M10	14.15±0.49	78.8±0.06	3.67±0.02	37.82±5.19	30.71±0.64	74.52±0.02	1.23±0.13
M11	40.24±0.23	0.1±0.008	11.89±0.04	34.78±0.22	25.54±0.29	60.18±0.02	1.36±0.009
M12	18.25±0.05	40.3±0.13	2.44±0.08	37.93±0.14	32.72±0.08	70.63±0.04	1.15±0.00
M13	22.57±0.04	18.2±0.05	5.63±0.25	38.14±0.68	31.16±0.12	69.31±0.01	1.22±0.01
M14	36.651±0.47	21.5±0.5	2.92±0.17	38.25±0.09	31.36±0.11	66.61±4.26	1.21±0.001
M15	33.17±0.23	1.2±0.2	5.51±0.04	37.65±0.49	25.38±0.06	63.01±0.02	1.48±0.008
M16	4.36±0.15	41.6±0.07	2.02±0.76	37.39±0.27	31.00±0.31	68.09±0.007	1.20±0.004
M17	27.51±0.64	10.7±0.25	2.22±0.19	40.68±0.39	30.06±0,08	71.04±0.05	1.35±0.006
M18	26.54±0.66	5.6±0.12	2.19±1.18	37.98±0.23	30.36±0.07	68.32±0.03	1.25±0.006
M19	5.52±0.072	554.6±0.2	2.72±0.14	37.39±0.12	36.44±0.21	73.81±0.02	1.02±0.001
M20	26.73±0.05	0.6±0.05	5.00±0.01	36.65±0.067	29.56±0.99	66.21±0.01	1.24±0.02
M21	4.59±0.17	7,81±0.06	3.70±0.24	37.88±0.017	29.37±0.04	67.22±0.03	1.28±0.00
M22	9.25±0.50	29,7±0.08	1.57±0.14	42.39±2.35	36.89±0.42	79.29±0.007	1.14±0.04
M23	8.88±0.06	21,8±0.16	2.68±0.14	37.55±0.04	32.28±0.04	69.82±0.03	1.16±0.00
M24	14.33±0.16	11,6±0.18	1.20±0.23	40.93±0.05	32.64±0.04	73.54±0.05	1.25±0.001
M25	33.4±0.2	21,5±0.22	3.91±0.17	37.37±0.50	30.80±0.93	68.18±0.02	1.21±0.01
M26	45.3±10.78	1,12±0.16	6.27±0.10	39.41±0.48	26.48±0.26	65.90±0.007	1.48±0.002
M27	00	303,5±0.45	14.93±0.28	29.33±0.02	31.98±0.07	61.36±0.05	0.91±0.001
M28	10.72±1.94	5,7±0.05	5.90±0.32	31.94±0.01	30.71±0.06	62.43±0.3	1.04±0.001
M29	0.86±0.02	263,4±0.06	12.39±0.25	31.22±0.15	31.67±0.12	62.74±0.2	0.98±0.000
M30	15.80±0.29	28,8±0.85	2.98±0.11	36.23±0.01	29.56±0.10	65.78±0.02	1.22±0.001
M31	10.85±0.22	8,3±0.05	4.00±0.17	36.21±2.99	30.91±0.58	68.66±0.65	1.17±0.07
M32	14.87±0.17	16,8±0.09	2.49±0.16	36.61±0.02	28.05±0.21	64.88±0.31	1.30±0.006
M33	22.18±0.65	15,2±0.05	7.11±0.78	38.40±1.34	31.17±0.12	69.59±0.01	1.23±0.02
M34	0.11±0.005	514,3±0.26	6.02±0.24	33.69±0.79	37.079±0.03	70.79±0.01	0.90±0.01
M35	14.97±0.22	18,5±0.16	2.51±0.18	41.99±0.13	31.68±0.36	73.68±0.02	1.32±0.007
M36	0.12± 0.10	517,03±0.05	6.30±0.14	33.33±0.06	37.65±0.00	71±0.00	0.88±0.001
Min	00	0.170	1,20	29.33	25.38	60.18	0.88
Max	45.3	571.9	14.93	42.39	37.65	79.29	1.54
Limits of international standards (Codex, EU)							
	≥8	40	≤5	No fixed limit	No fixed limit	≥60	No fixed limit

studied Algerian honeys (Ouchemoukh et al., 2007; Khalil et al., 2012).

The fructose/glucose ratio ranged between 0.88 and 1.54 with an average of 1.19± 0.15, indicating their floral origin because it is known that flower honeys have a fructose/glucose ratio of about 1 while in honeydew honeys the ratio ranges between 1.5 and 2.0 (Gleiter et al., 2006; Kivima et al., 2014).

In addition, the fructose/glucose ratio was calculated for all the 36 honey samples. This ratio tells about the

Table 2. Results of antibacterial substances.

Parameter	Number of samples	Results	Limits of international standards (Codex)
Search of Tetracycline	34	No residues of tetracyclin	No fixed limit
Search of oxytetracycline	2	0.03 ppb	No fixed limit
Search of Streptomycin	36	No residues of streptomycin	No fixed limit

crystallization state of honey, that is, when fructose is higher than glucose the honey is fluid (Ouchemoukh et al., 2010). Eventually, 30 examined Algerian honey samples were fluid (ratio great than 1).

The sucrose content of the honey samples analyzed in this study varied between 1.18 - 14.9 g/100 g with an average value of 4.55± 0.12. However 11 samples are not in conformity with international standard (≤5 g/100 g). The high content of this sugar means most of the time, an early harvest of the honey, that is, a product in which the sucrose has not been fully transformed into glucose and fructose by the action of invertase. This value indicates probably that the beekeeper use sucrose syrup to over feeding the bees in the winter season (Chefrour et al., 2009).

Honey is generally considered a natural and healthy food without additives or other foreign substances according to Directive 2001/110/CE. However, in the last decade the results of residue analyses carried out on presently marketed honey have changed the situation revealing what was known by many (Bogdanov, 2006).

The aim of this study was to clarify the situation concerning antibiotic residues in some Algerian honey (Table 2). Analysis performed by the laboratory to detect residues of tetracycline by LC/MS and streptomycin by HPLC in honey have revealed only insignificant traces of oxytetracycline (Figure 3a & b) in two samples of honey (0.03 ppb). All the other samples were negative for both antibiotics (Table 2, Figure 3).

Accordingly, in Europe a lack of harmonization among different countries on this matter is registered. For instance, in Belgium the action limit for antibacterial substances is fixed at 20 ng/ g. In Switzerland and the UK, the action limit applied for tetracycline (TCs) is 20 ng/g, respectively (Bogdanov, 2006; Baggio et al., 2009). Given the absence of maximum residue limits (MRLs) set for honey, the detection threshold was considered the threshold of positivity: 3 - 30 ng/g for tetracycline and from 5 - 200 ng/kg for streptomycin. Antibiotics are used in apiculture as anti-bacterial foulbrood diseases, like American Foulbrood (AFB).

Antimicrobial drugs are effective against foulbrood diseases; however, antibiotic drug residues in honey pose a potential risk to human health. These antibiotic residues have toxic acute and chronic effects on human health and also reduce the efficacy and quality of honey (Zai et al., 2013; Ajibola et al., 2012). On the other hand, to protect the image of honey as a healthy natural product, these bactericides are banned from honey (Michel et al., 2004).

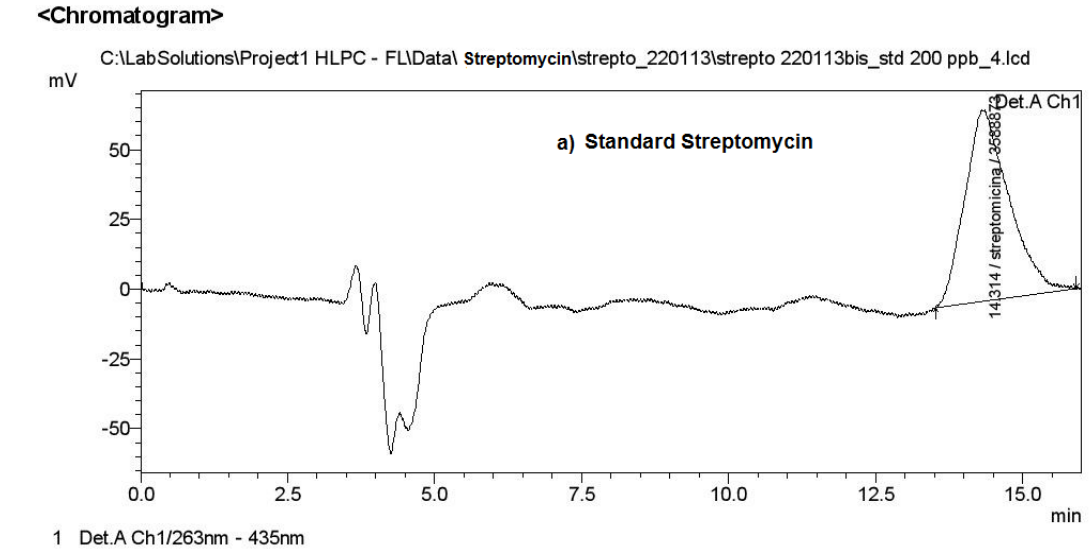
Conclusions

Thirty six Algerian honey samples were investigated for their physicochemical properties and research of streptomycin and tetracycline antibiotics. The study assessed the quality of honey samples analysed. In this study only 56% of samples were in agreement with the requirements of European Union and Codex Alimentarius Standards, while about 44% of them did not fit within European and Codex standards relative to the sucrose content, diastase activity and HMF reflecting inadequate sample manufacture and/or storage and adulteration. On the other hand, it was also concluded from this study that streptomycin and tetracycline were not used by the Algerian beekeepers for curing bacterial honeybee diseases.

According to the results some consideration may be given to Algerian beekeepers:

(i) It will be necessary to create more effective extension service to improve the beekeepers knowledge on honey harvesting techniques, honey processing and storage technologies.
(ii) Also, there is the need to increase the educational activity addressed to the beekeepers about taking care of Algerian honey production with special reference to physico-chemical characteristics, and have more responsibility for the quality of honey to be placed on the market with the appropriate label comprising the floral origin and chemical composition of honey.
(iii) There is the need to use reliable methods of control in order to ensure the conformity of honey product to avoid any risk of falsification and adulteration of Algerian honeys.

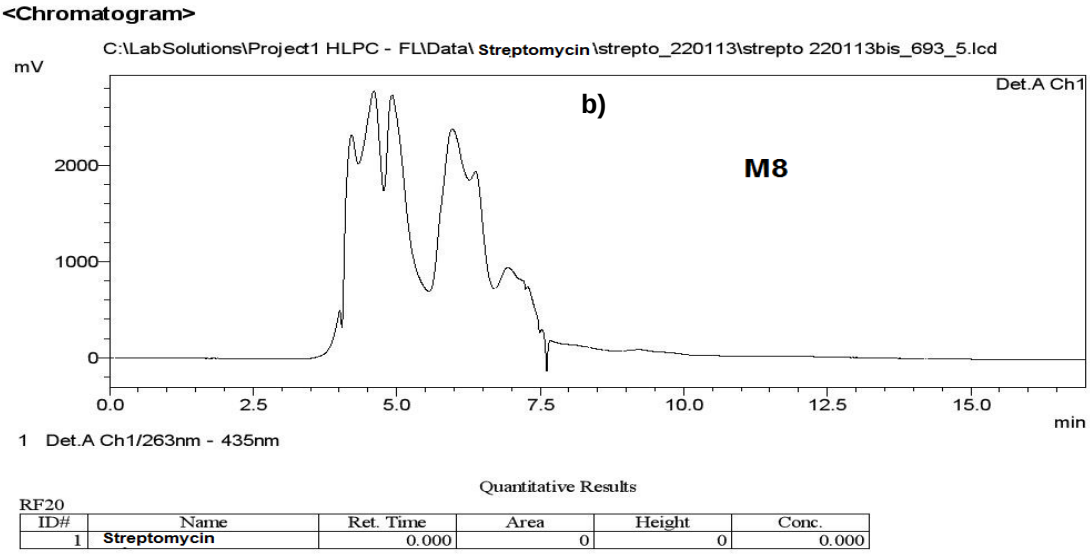
Moreover, a good knowledge of the Algerian product would provide the scientific support for the introduction of a national norm for honey.



Quantitative Results

RF20

ID#	Name	Ret. Time	Area	Height	Conc.
1	**Streptomycin**	14.314	3588873	68584	280.276



Quantitative Results

RF20

ID#	Name	Ret. Time	Area	Height	Conc.
1	**Streptomycin**	0.000	0	0	0.000

Figure 3. Chromatograms of Streptomycin by HPLC in Algerian honey (**a**) standard of streptomycin. (**b**) Example of an Algerian honey exempt of streptomycin.

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Conflict of interest

The authors declare no conflict of interest.

REFERENCES

Ajibola AW, Joseph PC, Kennedy HE (2012). Nutraceutical values of natural honey and its contribution to human health and wealth. J.

Nutr. Metab. 61(9): 1743-7075.
Ajlouni S, Sujirapinyokul P (2010). Hydroxymethyl furfuraldehyde and amylase contents in Australian honey. Food Chem. 119(3):1000-1005.
Albino G, Cristiana B, Biancotto G, Baggio A, Dainese N, Manzinello C, Mutinelli F (2005). Antibiotic residue in honey: alidation procedure. Honey analytical methods validation. *Apiacta*. 40:45-49.
Anklam E (1998). A review of the analytical methods to determine the geographical and botanical origin of honey. Food Chem. 63: 549-562.
Baggio A, Gallina A, Benetti C, Mutinelli F (2009). Residues of antibacterial drugs in honey from the Italian market. Food Additives and Contaminants: Part B. 2(1):52-58.
Ball WD (2007). The chemical composition of honey. J. Chem. Edu. 84:1643.
Bendeddouche B, Dahmani K (2011). Physical properties of honey products in Algeria. J. Stored Prod. Postharvest Res. 2:237-244.
Blasa M, Candiracci M, Accorsi A, Piacentini MP, Albertini MC, Piatt E (2006). Raw millefiori honey is packed full of antioxidants. Food Chem. 97: 217-222.
Bogdanov S (2002). Harmonised Methods of the International Honey Commission. International Honey Commission, 1-62 pp.
Bogdanov S (2006). Contaminants of bee product. Apidologie 37: 1-18.
Buba F, Gidado A, Shugaba A (2013). Physicochemical and Microbiological Properties of Honey from North East Nigeria. Biochem Anal. Biochem. 2(142):1-7.
Cara CM, Dumitrel GA, Glevitzky M, Perju D (2012). Stability of tetracycline residues in honey. J. Serb. Chem. Soc. 77:879-886.
Chakir A, Romane A, Marcazzan GL, Ferrazzi P (2011). Physicochemical properties of some honeys produced from different plants in Morocco. Arabian J. Chem. doi:10.1016/j.arabjc.2011.10.013
Chefrour A, Draiaia R, Tahar A, Ait Kaki Y, Bennadja S, Battesti M J (2009). Physicochemical characteristics and pollen spectrum of some North-east Algerian honeys. Afr. J. Food Agric. Nutr. Dev. 9:1276-1293.
Chua LS, Adnan NA, Abdul-Rahaman NL, Sarmidi MR (2014). Effect of thermal treatment on the biochemical composition of tropical honey samples. Inter. Food Res. J. 21(2):773-778.
Chua LS, Adnan NA (2014). Biochemical and nutritional components of selected honey samples. Acta Sci. Pol. Technol. Aliment. 13(2):169-179.
Coco FL, Valentini C, Novelli V, Ceccon L (1996). High-performance liquid chromatographic determination of 2-furaldehyde and 5-hydroxymethyl-2-furaldehyde in honey. J. Chromatogr. A. 95-102.
Codex Alimentarius. Commission Standards, Codex Standards for Honey, (1981/ revised 1987/revised 2001) (2001). FAO-Rome. pp. 1-7.
Council Directive of the European Union. Council Directive 2001/110/EC of 20 December 2001 relating to honey (2002). Official J. Eur. Communit. L10:47-52.
Cristofani E, Antonini C, Tovo GR, Fioroni L, Piersanti A, Galarini R (2009). A confirmatory method for the determination of tetracyclines in muscle using high-performance liquid chromatography with diode-array detection. Analytica Chimica Acta. 637:40-46.
Escuredo O, Seijo MC, Fernandez-Gonzalez M (2011). Descriptive analysis of *Rubus* honey from the north-west of Spain. Int. J. Food Sci. Tech. 46:2329-2336.
Fallico B, Zappala M, Arena E, Verzera A (2004). Effects of conditioning on HMF content in unifloral honeys. Food Chem.. 85: 305-313.
Glatt H, Schneider H, Liu Y (2005). V79-hCYP2E1-hSULT1A1, a cell line for the sensitive detection of genotoxic effects induced by carbohydrate pyrolysis products and other food-borne chemicals. Mutation Res. 580:41-52.
Islam MN, Khalil MI, Asiful MI, Gan SH (2014). Toxic compounds in honey. J. Appl. Toxicol. 34: 733-742.
Jilani IBH, Schweizer P, Khouja ML, Zouaghi M, Ghrabi Z (2008). Physicochemical spectra of honeys produced in Tunisia (Southwest of Kef). Apiacta 43:38-48.
Kebede N, Subramanian PA, Gebrekidan M (2012). Physico-chemical analysis of Tigray honey: An attempt to determine major quality markers of honey. Bull. Chem. Soc. Ethiop. 26: 127-133.
Khalil MI, Moniruzzaman M, Boukraâ L , Benhanifia M, Asiful MI, Nazmul IM, Amrah SS, Siew Gan H (2012). Physicochemical and Antioxidant Properties of Algerian Honey. Molecules**.** 17(9): 11199-11215.
Khalil MI, Sulaiman SA, Gan S H (2010). High 5-hydroxymethylfurfural concentrations are found in Malaysian honey samples stored for more than one year. Food Chem. Toxicol. 48: 2388-2392.
Kivima E, Seimanb A, Palla R, Sarapuua E, Martverka K, Laos K (2014). Characterization of Estonian honeys by botanical origin. Proceedings of the Estonian Academy of Sciences. 63(2): 183-192.
Kowalski S, Lukasiewicz M, Aleksandra DC, Gabriela Z (2013). 5-Hydroxymethyl-2-Furfural (HMF) – Heat-Induced Formation, Occurrence in Food and Biotransformation – a Review. Pol. J. Food Nutr. Sci. 63: 207-225.
Kubis I, Ingr I (1998). Effects inducing changes in hydroxylmethylfurfural content in honey. Czech J Animal Sci. 43: 379-383.
Kwapong PK, Ilechie AA, Kusi R (2013). Comparative antibacterial activity of stingless bee honey and standard antibiotics against common eye pathogens. J. Microbiol. Biotechnol. Res. 3: 9-15.
Makawi SZA ,Taha MI, Zakaria BA, Siddig B, Mahmod H, Elhussein AR, Elrasheed AGK (2009). Identification and Quantification of 5-Hydroxymethyl Furfural HMF in Some Sugar-Containing Food Products by HPLC. *Pak* . J. Nutr. 8:1391-1396.
Makhloufi C, Kerkvliet JD, D'albore RG, Choukri A, Samar R (2010). Characterization of Algerian honeys by palynological and physico-chemical methods. Apidologie, 41: 509-521.
Manzanares AB, Garcia ZH, Galdon BR, Rodriguez ER, Romero CD (2011). Differentiation of blossom and honeydew honeys using multivariate analysis on the physicochemical parameters and sugar composition. Food Chem. 126:664-672.
Marchini LC, Moreti ACCC, Ivani PO, Sodré GS (2007). Physicochemical composition of *Apis mellifera* honey samples from São Paulo State, Brazil. Quim. Nova. 30: 1653-1657.
Michel VB, Stef JMO, Klaas MJ, Enne DB (2004). Determination of streptomycin and dihydrostreptomycin in milk and honey by liquid chromatography with tadem mass spectrometry. J. of chromatography A, 1058: 137-142.
Ouchemoukh S, Louaileche H, Schweitzer P (2007). Physicochemical characteristics and pollen spectrum of some Algerian honeys. Food Cont. 18: 52-58.
Ouchemoukh S, Schweitzer P, Bey MB, Djoudad-Kadji, H, Louaileche H (2010). HPLC sugar profiles of Algerian honeys. Food Chem. 121:561-568.
Oudjet k (2012). Le miel: une denrée a promouvoir .Infos-CACQE 00:1-3.
Persano-Oddo L, Baldi E, Accorti M (1990). Diastatic activity in some unifloral honeys. Apidologie 21:17-24.
Popa M, Roxana B, Simona V, Mirel G, Elena B (2009). Study regarding the quality of honey-changes in hydroxymethylfurfuraldehyde (HMF) content of honey from Transylvania (Romania). *Annals.* Food Sci. Technol. 10:506-509.
Sahinler N, Sahinler S, Gul A (2004). Biochemical Composite ion of Honeys Produced in Turkey. *J.* Apic. Res. 43:53-56.
Sak-Bosnar M, Sakac N (2012). Direct potentiometric determination of diastase activity in honey. Food Chem.135: 827-831
Saxena S, Gautam S, Sharma A (2010). Physical, biochemical and antioxidant properties of some Indian honeys. Food Chem. 118:391-397.
Silva ADS, Alves CN, Fernandes KDG, Müller RCS (2013). Classification of honeys from Pará State (Amazon region, Brazil) produced by three different species of bees. J. Braz. Chem. Soc. 24:1135-1145.
Singh N, Bath PK (1998). Relationship between heating and
Singh MP, Hemant RC, Manish A, AKHIL M , Mukesh S, Deepak S, Sheeba K (2012). Honey as complementary medicine: - A review. Int. J. Pharma Biol. Sci. 3:12-31.

hydroxymethylfurfural formation in different honey types. J. Food Sci. Technol. 35: 154-156.
Sommer Y, Hollnagel H, Schneider H, Glatt H (2003). Metabolism of 5-hydroxymethyl-2-furfural (HMF) to the mutagen 5-sulfoxymethyl-2-furfural (SMF) by individual sulfotransferases. Naunyn-Schmiedeberg's. Arch. Pharmacol. 367: 166.
Vit P, Pulcini P (1996). Diastase and invertase activities in Meliponini and Trigonini honeys from Venezuala. J. Apicul. Res. 35 (2): 57-62.
White JW (1992). Quality Evaluation of Honey. Role of HMF and Diastase Assay. *Am. Bee J.* 132: 737-743, 792-794.
White JW, Riethof M, Subers M, Kushmir I (1962). Composition of American honey. USDA Technol. Bull. 1261:1-124.
Zai I U M, Khaliqur R, Arshad H, Shafqatullah (2013). Detection and quantification of residues in honey samples by chromatographic Tech. *Middle- East J. of scientific Research*. 14: 683-687.
Zerrouk S, Boughediri L, Carmen MS , Fallico B, Arena E, Ballistreri G (2013). Pollen spectrum and physicochemical attributes of sulla (*Hedysarum coronarium*) honeys of Médéa region (Algeria). Albanian J. Agric. Sci. 12 (3): 511-517.

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