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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.

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Full Length Research Paper

Hemin transported protein of *Xanthomonas axonopodis* pv. *glycines* functions on leaf colonization and virulence on soybean

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***Xanthomonas axonopodis* pv. *glycines* (Xag) causes bacterial pustule disease on soybean. This bacterium is present worldwide around hot and humid growing regions such as Southeast Asia. To understand if the gene coding for hemin transport protein (*hem*) is involved in virulence of the pathogen in soybean, we generated a *hem* mutant in Xag by overlapping PCR mutagenesis. Disruption of *hem* significantly reduced the population size and the disease incidence when sprayed on soybean but not when injected directly to soybean. The *hem* mutant caused the hypersensitive response induction on tobacco as an Xag wildtype. Interestingly, the *hem* expression was also reduced when the Xag wildtype grow *in planta*. The hemin transporter protein involved in the production of extracellular polysaccharide, biofilm formation, motility and attachment but not for extracellular enzymes. This confirmed that epiphytic fitness of Xag strongly required *hem* functions. These results suggest that *hem* gene is essential for virulence of Xag on soybean during the infection process.**

Key words: Bacterial pustule disease, virulence factors, iron uptake system, *hem* gene, epiphytic fitness.

INTRODUCTION

Bacterial diseases of soybean appear worldwide and cause production losses and decreases yield by reducing quality and quantity. The most common bacterial disease of soybean is bacterial pustule, caused by *Xanthomonas axonopodis* pv. *glycines*, is one of the most serious diseases of soybean in several part of soybean production areas including Thailand. Bacterial pustule lesions are small pale green spots with raised centers on either or both leaf surfaces. The bacterial pustule lesions may enlarge and coalesce, leading to premature defoliation (Narvel et al., 2001). Severe disease causes yield losses up to 40% (Prathuangwong and Amnuaykit, 1989). *X. axonopodis* pv. *glycines* infects the soybean plant through stomata and wounds. After invasion into the plant, bacteria multiply within intercellular spaces of the spongy mesophyll for pustule induction on susceptible soybeans (Jones and Fett, 1985).

Nutritional conditions are reported to be an important virulence factors for the disease induction of plant pathogenic bacteria. For example, the translations of pathogenicity island (*hrp* gene cluster) of *Xanthomonas* are induced by sucrose and sulfur-containing amino

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acids (Schulte and Bonas, 1992). While iron is an essential element for pathogenic bacteria due to its participation in the tricarboxylic acid cycle, electron transport, amino acid and pyrimidine biosynthesis, DNA synthesis, and other critical functions (Lemanceau et al., 2009). Moreover, iron is considered to play a critical role in plant-bacterial interactions. During bacterial infection, there is aggressive competition between the plant and the bacteria iron can play a critical role in such competitive relationships (Lemanceau et al., 2009). Segond and collaborators found that metal transporter AtNRAMP3 in Arabidopsis is upregulated in leaves challenged with the *Pseudomonas syringae* and *Erwinia chrysanthemi* (Segond et al., 2009). Similarly, *A. thaliana* synthesizes the ferritin AtFER1, an iron storage protein, is required for Arabidopsis resistance to *E. chrysanthemi* infection (Boughammoura et al., 2007).

Iron is one of the factors that limits bacterial growth *in planta* because concentrations of iron are necessary to support bacterial growth and multiplication (Expert et al*.,* 1996). Siderophore-mediated transport of iron is one of the mechanisms used by bacteria to uptake iron from their environment (Braun et al., 1998; Lee, 1995; Mietzner and Morse, 1994). Thus, the production of a siderophore by bacterial pathogens could significantly deplete the iron reserves of the host plant and weaken host-defence reactions (Lemanceau et al., 2009). Several studies have shown the importance role of iron in virulence of plant pathogenic bacteria. For instance, siderophore-deficient mutants of *Erwinia amylovora*, *E. chrysanthemi* strain 3937, *Erwinia carotovora* subsp. *carotovora*, *Ralstonia solanacearum* and *Agrobacterium tumefaciens* are virulence deficient on its host plants (Dellagi et al., 1998; Franza et al., 2005; Bhatt and Denny, 2004; Bull et al., 1996; Rondon et al., 2004). However, *P. syringae* pv. *syringae* B301D and *P*. *syringae* pv. *tomato* DC3000 do not show any growth defect or alter virulence on host plant (Jones et al., 2007; Jones and Wildermuth; 2011). In addition to the uptake of iron, several *Xanthomonas* take up iron via *tonB* system. In *Xanthomonas campestris* pv. *campestris*, mutation of *tonB*, *exbB* and *exbD1* genes which are involved in iron uptake system have been reported to be impaired for ferric ion uptake and exhibited reduced virulence in cabbage (Wiggerich and Puhler, 2000). The *fur* mutant of *Xanthomonas oryzae* pv. *oryzae* is virulence deficient and hypersensitive to oxidative stress (Subramoni and Sonti, 2005). Different pathways of iron uptake from direct Fe^{2+} transport and host iron binding proteins or heme also may be employed by pathogenic bacteria (Ratledge and Dover, 2000; Velayudhan et al., 2000).

Hemin is one of heme oxidized form that consists of an iron ion and found in extracellular environments (Lee, 1995). Hemin iron transport and utilization systems have been identified in numerous bacterial species, where it was shown that an outer membrane receptor and a periplasmic binding protein-dependent ABC-type transporter are required for hemin uptake (Stojiljkovic and Hantke, 1992, 1994). It is the cofactor in reactions involved in various cellular functions including oxygen transport and electron transfer (Lee, 1995).

For phytopathogenic bacteria, *Xylella fastidiosa* 9a5c contains 67 genes encoding proteins involved iron metabolism and has been reported to contain five membrane receptors, including siderophore, ferrichrome-iron and hemin receptors, all of which are thought to be associated with iron transport, utilization and virulence (Simpson et al., 2000). Whereas, the extensive genetic and genomic resources are available for *X. axonopodis* pv. *glycines*, and it has been the subject of highly productive research centered on the mechanisms of plant host susceptibility/resistance and pathogen virulence and avirulence determinants (Athinuwat et al., 2009; Chatnaparat et al., 2012; Kasem et al., 2007; Kaewnum et al., 2005; 2006; Thowthampitak et al., 2008). Recently, the draft genome of *X. axonopodis* pv. *glycines* 12-2 has been sequenced and found that this strain contains genes encoding hemin uptake locus. Although, the effects of iron uptake system in cell growth and virulence production of several bacteria plant pathogens have been documented, hemin transport protein (*hem*) of the bacteria that involved in the infection process of plant are not established. Thus, in this study, a *hem* mutant was constructed using overlapping extension mutagenesis. The roles of *hem* in contribute to full virulence of Xag on soybean were investigated.

MATERIALS AND METHODS

Bacterial strains, plasmids and recombinant techniques

Bacterial strains and plasmids used in this study are described in Table 1. *X. axonopodis* pv. *glycines* wildtype strain 12-2 was cultured at 28°C in nutrient glucose agar (NGA) (Sambrook et al., 1989). Mutants were cultured on NGA containing 50 μg/ml kanamycin and 50 μg/ml chephalexin. The complemented *hem* was cultured on NGA containing 50 μg/ml kanamycin, 50 μg/ml cephalexin, and 40 μg/ml gentamycin. All DNA manipulations including DNA isolation, plasmid extraction, restriction digestion, ligation, and gel electrophoresis were performed as described previously (Sambrook et al., 1989).

Knockout of *hem* genes in *X. axonopodis* pv. *glycines* 12-2

A disruption of gene coding for hemin transport protein (*hem*) was accomplished using overlap extension mutagenesis (Figure 1). The upstream and downstream regions of *hem* gene in *X. axonopodis* pv. *glycines* 12-2 were amplified using HemKO-1-F and HemKO-1-R primers that unique to upstream region and HemKO-2-F and HemKO-2-R primers that unique to downstream region of *hem* gene respectively (Table 2), with one having an extension complementary to the kanamycin resistance cassette from pKD13 to generate two amplicons with ends overlapping those of the resistance cassette (Datsenko and Wanner, 2000). Overlap

Table 1. Bacterial strains and plasmids.

Bacterial strain or plasmid	Relevant characteristic	Reference or source
X. axonopodis* pv. *glycines		
12-2	Wildtype, soybean pathogen	Thowthampitak et al. (2008)
hem mutant	Km^r, *hem*-::Kan, 12-2 derivative	This study
hem+	*Hem* mutant complemented with pBBR::*hem*	This study
Plasmids		
pTok2	ColE1 replicon, suicide plasmid, Tc^r	Kitten and Willis, 1996
pKD13	FRT-Km^r-FRT, oriR6K, Ap^r, Km^r	Datsenko and Wanner (2000)
pBBR1MCS-5	Broad host range cloning vector, *lacZ*, Gm^r	Kovach et al. (1995)
pTok2::Δ *hem*	Δ *hem*::Kan from overlapping PCR cloned into pTok2, Tc^r, Km^r	This study

extension PCR was used to link the two PCR amplicons and the resistance cassette; this larger fragment was then cloned into the destination vector pTok2 using the quick ligation protocol (New England Biolabs Inc.) and introduced into *E. coli* S17-1, the mobilizing strain by transformation, then transferred to *X. axonopodis* pv. *glycines* via conjugation, selecting for transconjugants on NGA containing 50 μg/ml kanamycin and 50 μg/ml chephalexin as a sensitive antibiotic for *E. coli* strain. Gene disruption was confirmed using PCR, with primers specific (Hem-C-F and Hem-C-R) to the sequences flanking of *hem* gene (Table 2).

Complementation of *hem* mutants

To complement the *hem* mutant, 800 bp of *hem* containing the native promoter was amplified using primers *hem* com-F and *hem* com-R (Table 2). The amplicon was digested with *Hin*dIII and ligated into the multiple cloning site of vector pBBR1MCS-5 to yield pBBR::*hem,* which was then introduced into *hem* mutant by electroporation. The complemented *hem* mutant was cultured on NGA containing 50 μg/ml kanamycin, 50 μg/ml cephalexin, and 40 μg/ml gentamycin and also confirmed by PCR using primers *hem* com-F and *hem* com-R.

Real-time quantitative reverse transcription PCR analysis

The expression of *hem* was determined by real-time qRT-PCR of cDNA isolated from both an Xag wildtype and a *hem* mutant grown in nitrogen yeast glycerol broth (NYGB) for 24 h as well as in *X. axonopodis* pv. *glycines* cells recovered from infected soybean plants. For *in planta* experiment, Xag cells in soybean were isolated from the leaves according to the method described by Yu et al. (2013). Briefly, the bacterial cells at 1×10^8 cfu/mL were introduced by vacuum infiltration into soybean leaves. Infiltrated plants were incubated for 4 days. A total of 150 to 200 leaves were collected cut into squares, and submerged in an acidic phenol RNA-stabilizing solution. The solution was filtered and centrifuged to harvest the bacterial pellets. Total RNA preparation for using in the real-time qRT-PCR was isolated with TRIzol (Invitrogen Life Technologies) from cells grown in NYGB and in *planta* using the method of Santiago-Vazquez and associates (2006). cDNA was generated from 1 μg of RNA using SuperScript II (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) and random hexamers. Real-time qRT-PCR was performed on 1 μg of the cDNA using LightCycler FastStart DNA Master PLUS SYBR Green I (Roche, Indianapolis, IN, U.S.A.) on a Roche Lightcycler II (Roche) following the manufacturer's specifications. The specific primer pairs in this experiment are list in the Table 2. An external standard curve was generated using purified *ihfA* (integration host factor A) DNA (Champoiseau et al., 2006). Melting curve analysis was used to verify amplification of a single product. The concentration of amplification products from negative controls (RNA samples to which no superscript was added) was undetectable in all cases, indicating a lack of interference from contaminating DNA.

High iron concentration sensitivity assay

To test for high iron concentration sensitivity, an Xag wildtype, a *hem* mutant and a complemented *hem* mutant were grown in NYGB at 28°C with shaking at 200 rpm to an optical density (OD) at 600 nm of 1.0 (OD_{600} = 1.0). Cultures were transferred to NYGB supplemented with $FeCl_3$ to a series of final concentrations at 0, 4, 5, and 6 mM, respectively and were incubated at 28°C with shaking at 200 rpm. After incubation for 24 h, the cell density was measured spectrometrically using a spectrophotometer and absorbance at OD_{600} was determined (Yang et al., 2007). The experiments were repeated three times with at least three replicates in each experiment.

The extracellular polysaccharide (EPS) production

EPS production of an Xag wildtype, a *hem* mutant and a complemented *hem* mutant was measured with some modification as described by Tang et al. (1991). Cultures were grown in NYGB containing 4% glucose at 28°C with shaking at 200 rpm for 5 days. EPS was precipitated from the culture supernatant with ethanol. Then EPS was dried at 80°C to constant weight and the difference between the two weights was used to estimate the production of EPS per millilitre culture. The experiments were repeated three times with at least three replicates in each experiment.

Biofilm formation

Cells of *X. axonopodis pv. glycines* strains taken from cultures grown on nitrogen yeast glycerol agar (NYGA) for 24 h were suspended in NYGB and cell suspension of each strain was added

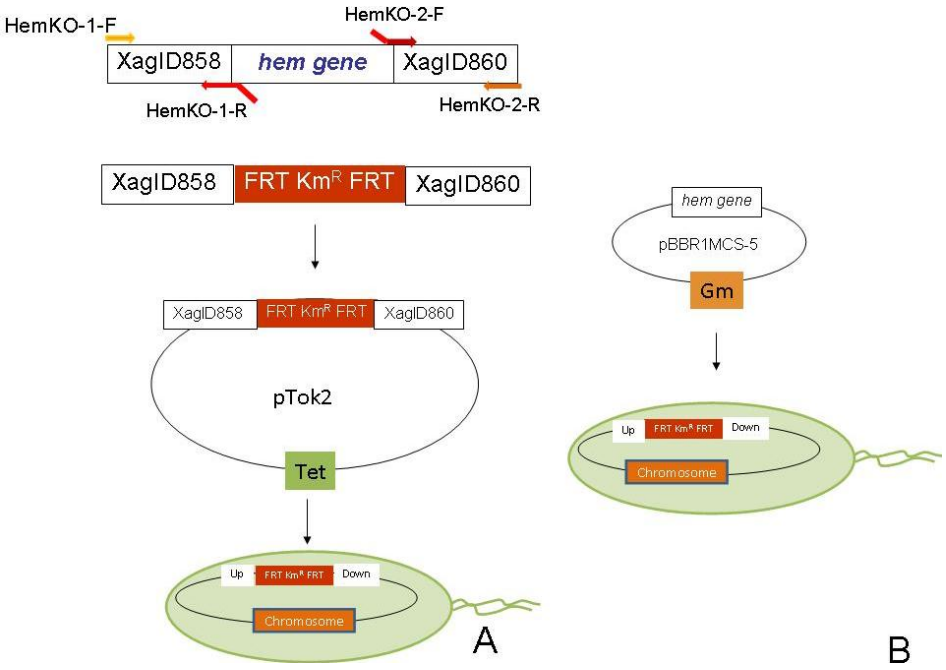

Figure 1. Overlap extension PCR was used to create constructs in the suicide-delivery vector pTOK2 to create site-directed mutant of *hem* gene in *Xanthomonas axonopodis* pv. *glycines* 12-2 by recombination (A). A *hem*-complementary strain**,** the 0.8-kb sequence of *hem* containing the native promoter was amplified and ligated into the multiple cloning site of vector pBBR1MCS-5(B).

Table 2. Polymerase chain reaction primers.

Primer	Sequence[a]	Description
HemKO-1-F	5' CGAAGACTATGGCAGCATC 3'	Amplification of upstream region of *hem* for generate *hem* mutant
HemKO-1-R	**5' GAAGCAGCTCCAGCCTACACA** GGTGTAATAGCTGTCCAGCTG 3'	……..
HemKO-2-F	5' GGTCGACGGATCCCCGGAAT GTTGTATCGCACTGACGCTG 3'	Amplification of downstream region of *hem* for generate *hem* mutant
HemKO-2-R	5' CTTCGAATACTGCCTGCAG 3'	……
hem com-F (*Hin*dIII)	5' TTGTAGTAAAGCTT ATTCGCGCCTGGCGCCAAG 3'	Amplification of 0.8 kb of *hem* for generate *hem* complementary strain
hem com-R (*Hin*dIII)	5' TTGTAGTAAAGCTT AGCGTCAGTGCGATACAACG 3'	……
Hem-C-F	5'CTGGCGGTGAGCTGGTAG 3'	Primers specific to the sequences flanking of *hem* gene
Hem-C-R	5'AGCGTCAGTGCGATACAAC 3'	……
*hem*RT-F	5'ACGAACTGGATGGCTGGCAGC 3'	Specific primer for *hem* expression using RT-PCR
*hem*RT-R	5' CGGCCGTCGATGCCCTCTTC 3'	……
*hrpF*RT-F	5' GACTCCATTTCCAAGGACGA 3'	Specific primer for *hrpF* expression using RT-PCR
*hrpF*RT-R	5' GCGCTCATGTTGTCGTAGAA 3'	……
*hrpD*RT-F	5' CGGTCACCCAAGATATGAGC 3'	Specific primer for *hrpD* expression using RT-PCR
*hrpD*RT-R	5' CATTGAAGTCGTTGCGTGAG 3'	……

[a]Bold sequence= Reverse complementary priming for pKD13 site, underlined sequence= pKD13 priming site.

to glass tubes, and grown at 28°C for three days. The presence of a biofilm was visualized as a white ring on the tube side wall, usually at the air-medium interface and quantified by crystal violet staining as previously described (Davey and O'Toole, 2000). Dye abundance was measured by absorption at 570 nm using a spectrophotometer. Readings from five replicates were averaged. The experiments were repeated three times with similar results.

Bacterial attachment

Bacterial adhesion of *X. axonopodis* pv. *glycines* strains to soybean leaves was assessed by immersing six individual leaves into 500 ml of a suspension of a given bacterial strain (10^7 cells/ml) at 28°C. After 5 min, 3 and 7 h, the leaves were removed and rinsed gently with distilled water for 30 s. To enumerate the attached bacteria a single 2 cm diameter disc was cut from a portion of each leaf in an area lacking major veins using a cork borer, the discs homogenized using a mortar and pestle, and cells enumerated by dilution plating on NYGA as in other studies (Chatnaparat et al., 2012). The experiments were repeated three times.

Motility analysis

Fresh colonies of an Xag wildtype, a *hem* mutant, and a complemented *hem* mutant from NYGA plates were stabbed into swarm and swimming plates composed of 0.03% (wt/vol) Bacto Peptone, 0.03% yeast extract, and 0.4% agar for swarm plate and 0.25% agar for swimming plate respectively. The inoculated cells were cultured for four days or longer at 28°C and examined for bacteria motile away from the inoculated site (Sockett and Armitage, 1991). The experiments were repeated three times and each experiment was measured in triplicate.

Extracellular enzymes assay

Relative levels of extracellular production including carboxymethyl-cellulase, α -amylase and protease were assessed by radial diffusion assays (Thowthampitak et al., 2008). The experiments were repeated three times and each experiment was measured in triplicate.

For carboxymethylcellulase production, inoculated plates containing an assay medium (0.1% carboxymethyl cellulose, 25 mM sodium phosphate, pH 7.0, and 0.8% agarose) were incubated at room temperature overnight, stained with 0.1% Congo red for 20 min, and washed twice with 1 M NaCl. Carboxymethyl cellulase (CMCase) activity was visualized as white halos surrounding the wells.

For α -amylase production, inoculated plates containing alpha-amylase assay medium (0.5% yeast extract, 1.0% tryptone, 0.25% NaCl, 0.2% soluble starch, and 0.8% agarose) were incubated at room temperature overnight and stained with potassium iodide for 10 min. α-amylase was detected as clear halos surrounding the wells.

For protease production, inoculated plates containing NYGA supplemented with 0.5% skimmed milk were incubated at room temperature for 48 h. Extracellular protease production was detected visually as clear halos surrounding the wells.

Hypersensitive response (HR) and virulence assay

The *X. axonopodis* pv. *glycines* strains were grown in NYGB at 28°C with shaking at 200 rpm. Cells were pelleted at early log phase by centrifugation at 6,000 rpm for 2 min. Cell pellets were suspended in sterile water for HR tests on tobacco. HR was assayed as described previously (Kaewnum et al., 2005). Briefly, tobacco plants were inoculated with bacterial suspensions (10^9 cells/ml) by injection of leaf with a syringe. Sterile demineralized water was used as a negative control. Infiltrated zones were observed for development of typical HR (tissue collapse and necrosis) for 24 to 48 h post-infiltration. The experiments were repeated three times with similar results.

The virulence of *X. axonopodis* pv. *glycines* strains was assessed on susceptible soybean cv. Spencer following topical spray application (Kaewnum et al., 2005). Briefly, cell suspensions of a given strain (OD_{600} = 0.2; ca. 10^8 cells/ml) in 1 mM KPO_4 buffer were sprayed onto leaves of plants (ca. 6 weeks old) maintained in a greenhouse (average temperature ca. 28°C). For the first 24 h after inoculation, plants were held in an enclosed plastic bag to maintain high humidity and moisture on leaves before being returned to the greenhouse bench. Three trifoliate leaves, collected each from the top, middle and basal portion of three plants from each of five replicate pots, were evaluated for each strain.

Cotyledon assay was done as described by Hwang et al. (1992). The 7-days old soybean seedling grown in a greenhouse was surface sterilized with 0.5% sodium hypochloride for 3 min and washed with sterile distilled water for 5 min. The cotyledon was punctured with sterile pins. 10 μl of each suspension of bacterial cells (10^8 cells/ml) of the wildtype, *hem* mutant, and complemented *hem* mutant were dropped on the wound site. Inoculated cotyledons were kept in high moisture conditions with 16h photo period at room temperature. The cotyledons were observed by chlorotic and necrotic symptoms around the inoculation site within 48 h after inoculation. At least five soybean cotyledons were used for each strain. The experiments were repeated three times with similar results.

Bacterial population on soybean leaf surface

For determination of epiphytic fitness, bacterial populations were isolated from the soybean leaves according to the modify method described by Morris et al. (1998). Inoculation of *X. axonopodis* pv. *glycines* strains on 6 weeks old soybean were designed to analyze the epiphytic fitness of the *hem* mutant in comparison to that of the Xag wildtype. Bacterial cultures were prepared to a final concentration of 4.5×10^5 cells per ml and then 50 ml of bacterial suspension was used to spray on soybean leaves as described above in virulence assay. Four pots containing 5 soybean plants in each pot were used for each strain. The plants were transferred to the greenhouse bench. Four leaves of inoculated soybeans were taken randomly from each treatment. Estimation of each bacterial numbers of the Xag wildtype, *hem* mutants, and complemented *hem* mutants were collected at 1, 3, 7 and 14 days after inoculation. The experiments were repeated two times.

RESULTS

Genetic characterization of the *hem* locus

Analysis of the DNA sequence of *X. axonopodis* pv. *glycines* 12-2 draft genome (GenBank accession number AJJO01000000) revealed the presence of the genes predicted as hemin uptake (*hem*) locus including hemin uptake protein, hemin uptake system outer membrane receptor, and hemin transport protein (*hem*), respectively. *hem* size is 800 bp encodes a protein of 211 amino acids.

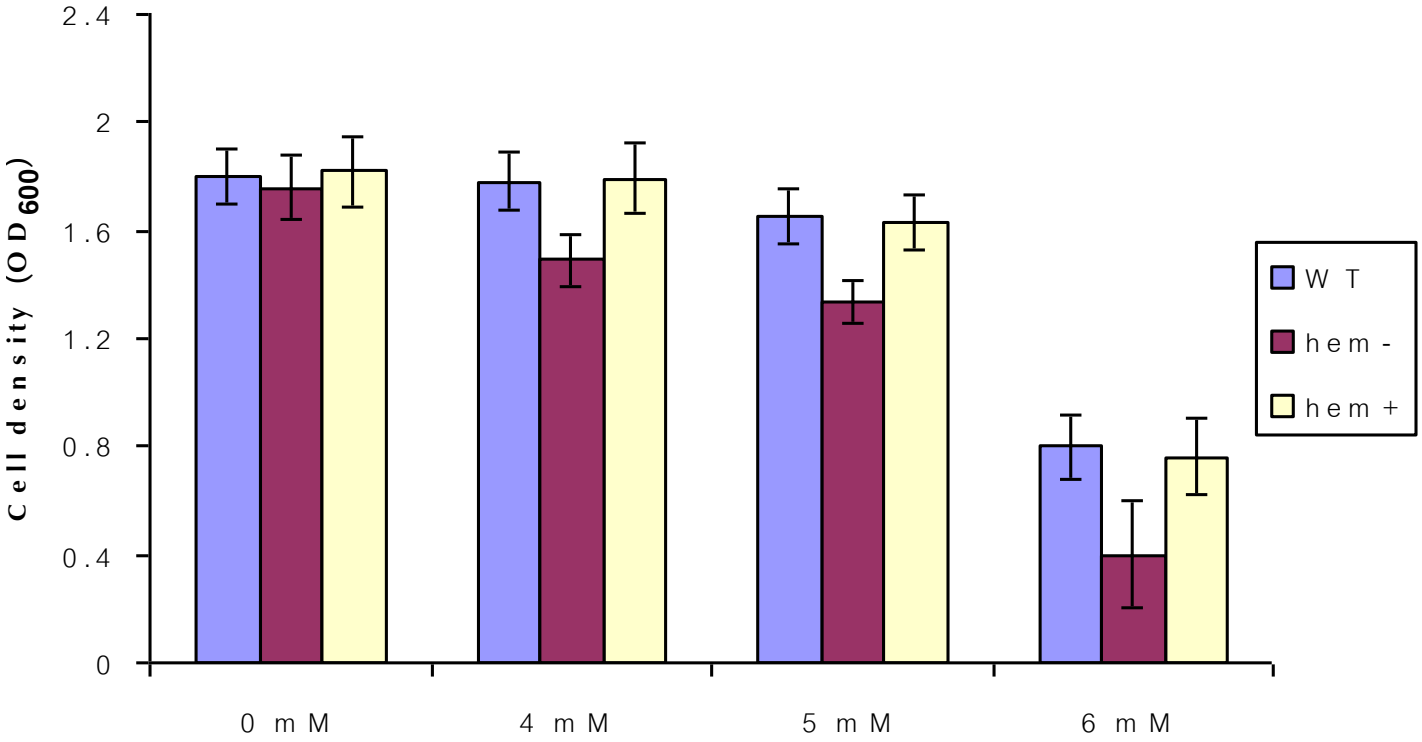

Figure 2. The growth of *Xanthomonas axonopodis* pv. *glycines* strains under different $FeCl_3$ concentration. Culture (10 µl, cell density adjusted to about 10^8 cells/ml) of each strains was inoculated into 5 ml of NYGB supplemented with different concentration of Fe^{3+} and were incubated at 28°C in shaker. WT = wildtype, *hem*$^-$ =*hem* mutant, and *hem*$^+$ = complemented *hem*. The cell density was measured spectrometrically at 600 nm after incubation for 24 h. Bars represent standard error of the means.

Hem shared the highest level of identity (99%) to the hypothetical protein of *Xanthomonas axonopodis* pv. *citri* 306 and *Xanthomonas citri* pv. *mangiferaeindicae* LMG 941, while it exhibited 80 and 70% identity to a hypothetical protein in *X. campestris* pv. *vesicatoria* 85-10 and *X. campestris* pv. *campestris,* respectively. However, protein predicted as hemin transport protein in *X. axonopodis* pv. *glycines* 12-2 shared similarity at 63 and 37% with hemin transport protein in *Stenotrophomonas maltophilia* D457 and *Sinorhizobium fredii* HH103 respectively.

High iron concentration sensitivity

The *X. axonopodis* pv. *glycines* wildtype and the *hem* mutant showed the same growth yield in NYGB without supplementation of $FeCl_3$. When NYGB supplemented with $FeCl_3$ to final concentrations of 0, 4, 5, and 6 mM, respectively, significant differences in growth were observed between the Xag wildtype and the *hem* mutant. The Xag wildtype could grow well in NYGB supplemented with concentrations up to 5 mM, whereas the *hem* mutant decreased the growth under all conditions (Figure 2). The growth capacity of the *hem* mutant could be completely restored in the complemented *hem* mutant.

Pathogenicity and virulence assay

Leaf pathogenesis assay was conducted to determine the probable involvement of *hem* functions in bacterial virulence. The *X. axonopodis* pv. *glycines* wildtype strain, the *hem* mutants, and the complemented *hem* mutant were inoculated through injection to soybean cotyledons and through spray on soybean leaves. All strains developed normal disease symptoms as in its wildtype when inoculated through injection into the soybean cotylendons (Figure 3B). Interestingly, the virulence of the *hem* mutant appeared to be attenuated when the cells were applied on soybean leaves by spray inoculation (Table 3, Figure 3A). Moreover, the hypersensitive response activities of the *hem* mutant were similar to that of the Xag wildtype after infiltration into tobacco. This results suggest that *hem* gene is essential for virulence of *X. axonopodis* pv. *glycines* on soybean before penetrate into soybean plant. Thus, perhaps *hem* gene is important for epiphytic fitness of this

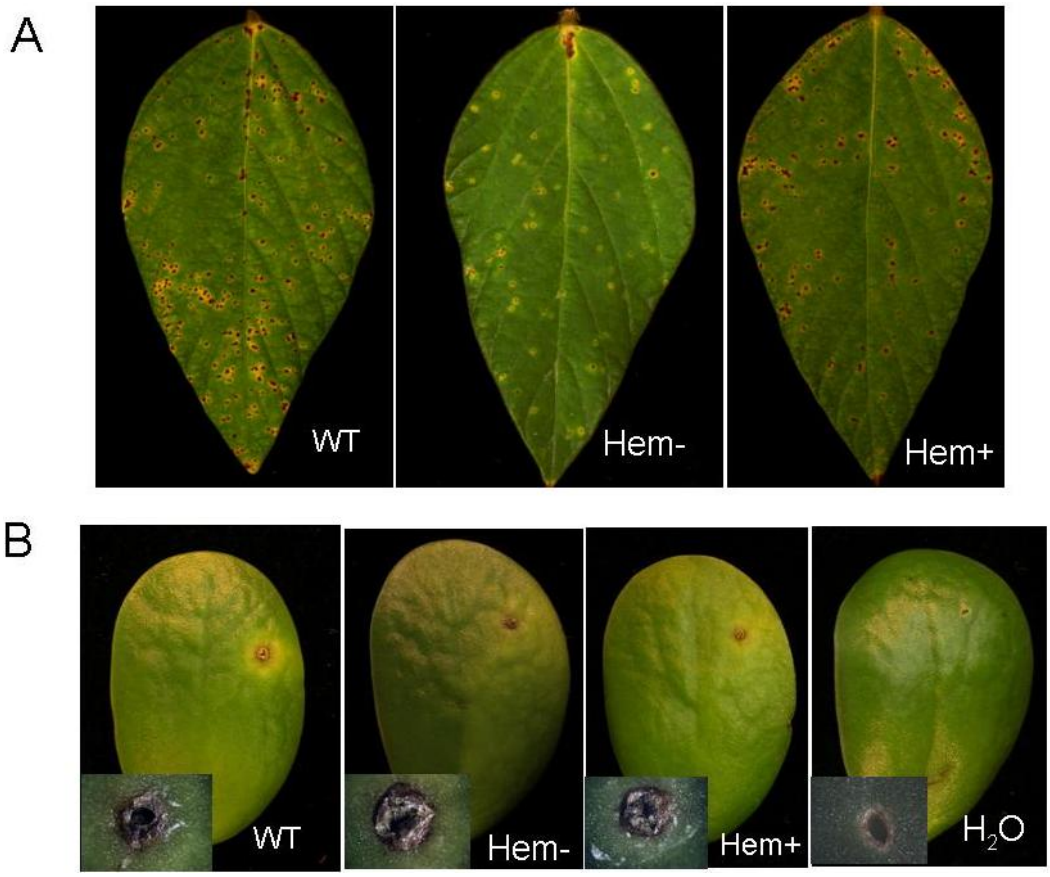

Figure 3. Virulence testing of the *Xanthomonas axonopodis* pv. *glycines* strains when bacterial cells were sprayed on soybean (A) and were injected into soybean cotyledons (B). WT = wildtype, *hem*$^{-}$ = *hem* mutant and *hem*$^{-}$ = *hem* complementary strain.

Table 3. Virulence deficient of *Xanthomonas axonopodis* pv. *glycines* by spray inoculation.

Strain	Mean of lesions per plant[a]	Relative virulence (%)
Xag12-2	260 ± 75	100
hem mutant	65 ± 23	25
hem complementary strain	246± 54	94

[a]Data shown are the averages ± standard deviations.

this pathogen.

hem gene expression

Real-time qRT-PCR was performed to determine transcript levels of *hem* gene in the *X. axonopodis* pv. *glycines* wildtype and the *hem* mutant in overnight culture in NYGB and also wildtype in soybean leaves after four days inoculation. Mutation in *hem* resulted in no expression of *hem* transcription in the culture medium confirming that the *hem* mutant strain completely lost *hem* gene. Furthermore, the level of *hem* transcript in the Xag wildtype cells recovered from infected soybean plants was 4.34 fold lower than that in the wildtype cells grown in culture (Figure 4). In addition, the expression of *hem* gene was not observed from cDNA of non-infected soybean. However, disruption of the *hem* gene did not affect the expression of the *hrpF* and *hrpD* genes, which are encoded in the *hrp* cluster when comparison with wildtype cells grown in Hrp inducing medium (data not shown). This results suggest that *hem* gene was down-

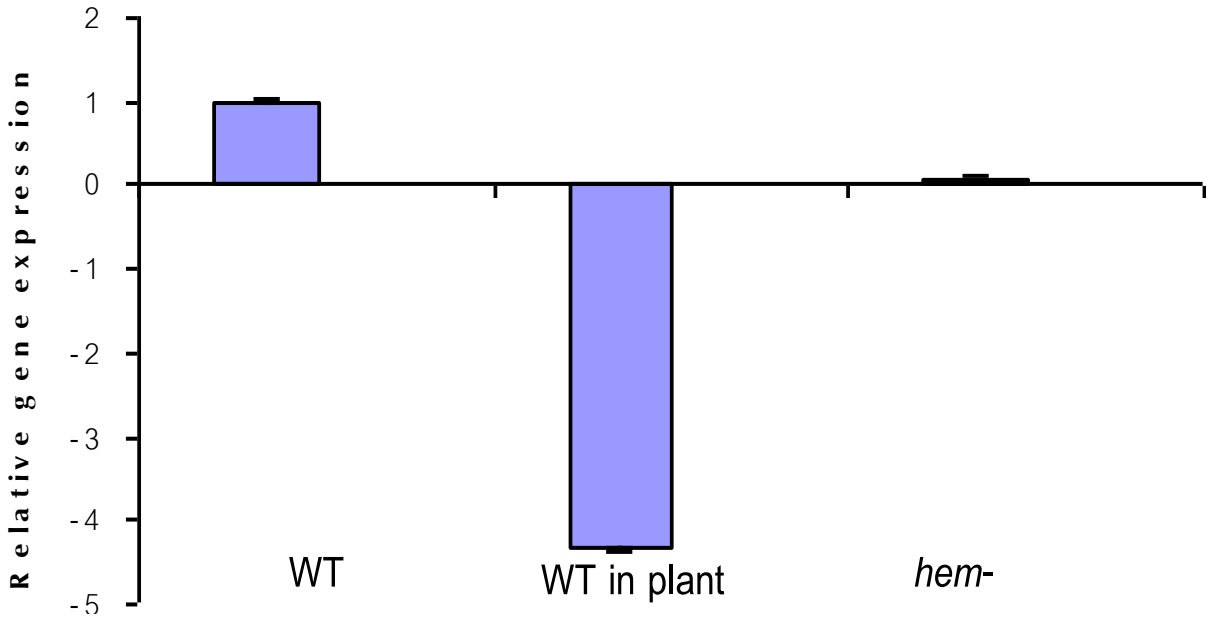

Figure 4. Relative abundance of transcripts of *hem* gene in *Xanthomonas axonopodis* pv. *glycines* wildtype (WT) and *hem* mutant (hem$^-$) grown in culture medium compared with wildtype in soybean plant as determined by real-time qRT-PCR. Vertical bars represent the standard error of mean ratio.

regulated when bacteria grow in soybean plant and did not affect typeIII secretion system of this pathogen in *vitro*. Therefore, the *hem* mutants show a virtually indistinguishable disease symptom when compared to that of the wildtype strain when the cells were directly injected into soybean (by pass epiphytic fitness phase).

Bacterial population on soybean

The above data suggest that *hem* gene contributed virulence on soybean leave when spray inoculation but not when injected into soybean. Therefore, we assessed the ability of the *hem* mutants to grow on soybean leaf surfaces. As we expected, minimal cells number were observed in soybean leaves sprayed with the *hem* mutants (Figure 5). Taken together, these data indicate that *hem* gene is required for the leaf colonization of Xag on soybean.

Extracellular polysaccharide (EPS) production

The EPS production in three-day liquid cultures showed that *hem* mutants produced on average 0.62 mg per milliliter of the culture, compared with 1.0 and 1.1 mg per milliliter of the culture of wildtype and complemented *hem* mutants, respectively (Figure 6). EPS is an important virulence factor in *X. axonopodis* pv. *glycines* and in many pathogens (Braun, 1990; Thowthampitak et al., 2008). Our results showed the *hem* mutants decreased the production of EPS. Thus, we assumed that the reduction in the production of EPS may contribute to the deficiency in virulence of the mutant.

Biofilm formation

Biofilm formation is structure for protect the bacterial cell from stress environmental condition. Plant pathogenic bacteria within biofilms are generally better resistant to environmental stress and host defense response (Crossman and Dow, 2004).The biofilm formation of the *hem* mutants in NYGB as measured by crystal violet staining after 3 days of incubation was significantly lower than that of the wildtype and complemented *hem* mutants (Figure 7). Therefore, the *hem* mutants may not resistant to environmental stress such as dry conditions or UV and host defense response during pathogenesis.

Bacterial attachment

Since we found that *hem* gene effected to biofilm formation on abiotic surface. Therefore, the attachment of *hem* mutants to the soybean leaves surface was studied by quantifying those cells remaining on leaves after they were dipped into bacterial cell suspensions. The number

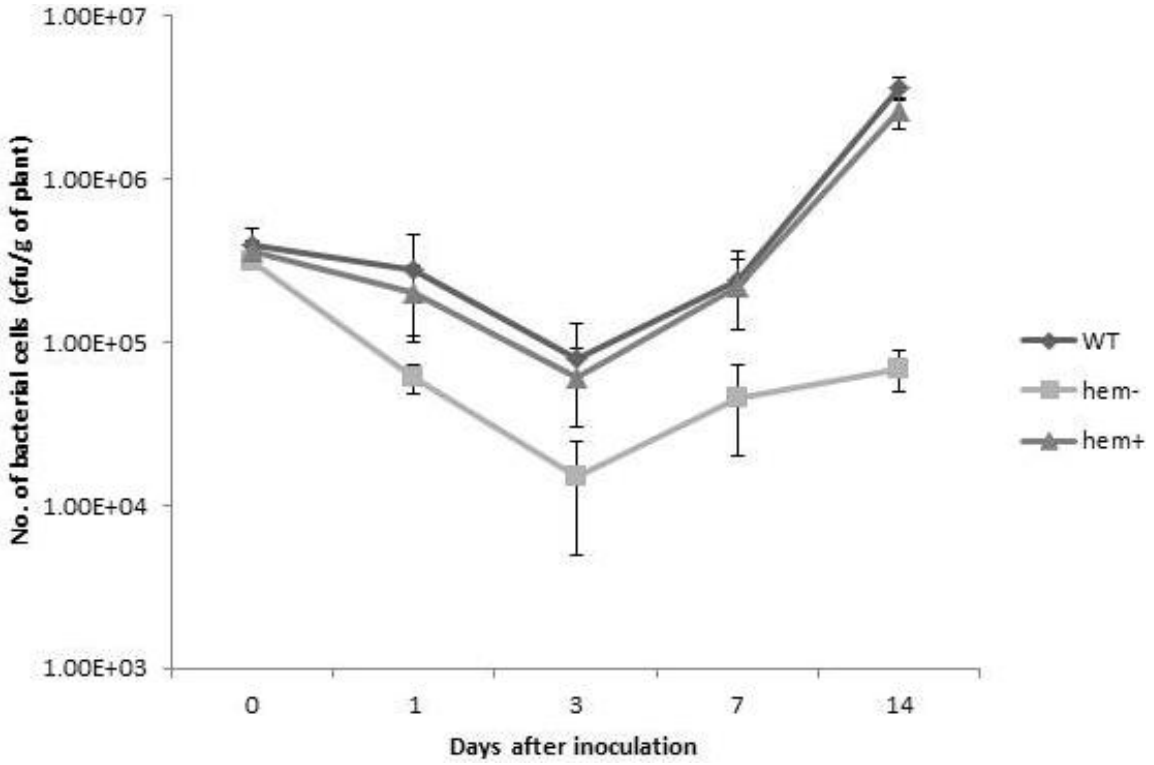

Figure 5. Bacterial population of *Xanthomonas axonopodis* pv. *glycines* strains on soybean leave surface after inoculation of each strain, at a concentration of 4.5×10^5 cells ml^{-1}, onto soybean leaves in a greenhouse experiment. WT = wildtype, *hem*$^-$ = *hem* mutant and *hem*$^-$ = *hem* complementary strain.

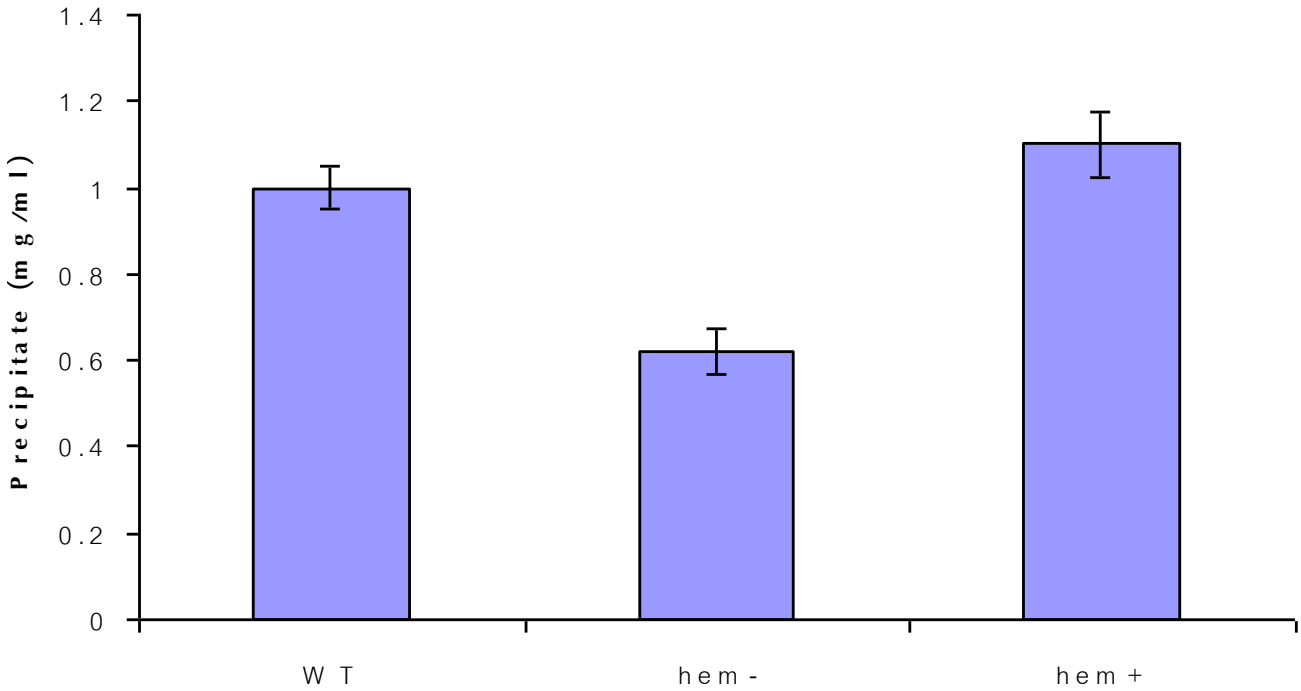

Figure 6. The *hem* mutant of *Xanthomonas axonopodis* pv. *glycines* affects the production of extracellular polysaccharide (EPS). WT = wildtype, *hem*$^-$ =*hem* mutant, and *hem*$^+$ = complemented *hem*. Bars represent the standard error of the means.

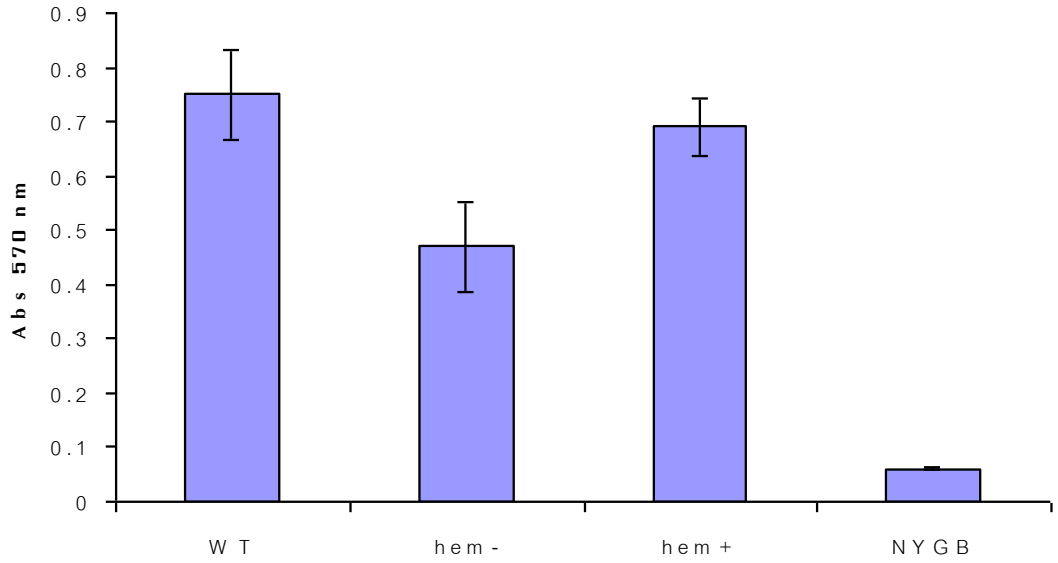

Figure 7. Biofilm formation on glass tube surfaces were assessed by the use of absorbance; WT = wild-type, *hem*$^{-}$ =*hem* mutant, *hem*$^{+}$ = complemented *hem*, and NYGB = liquid medium only.

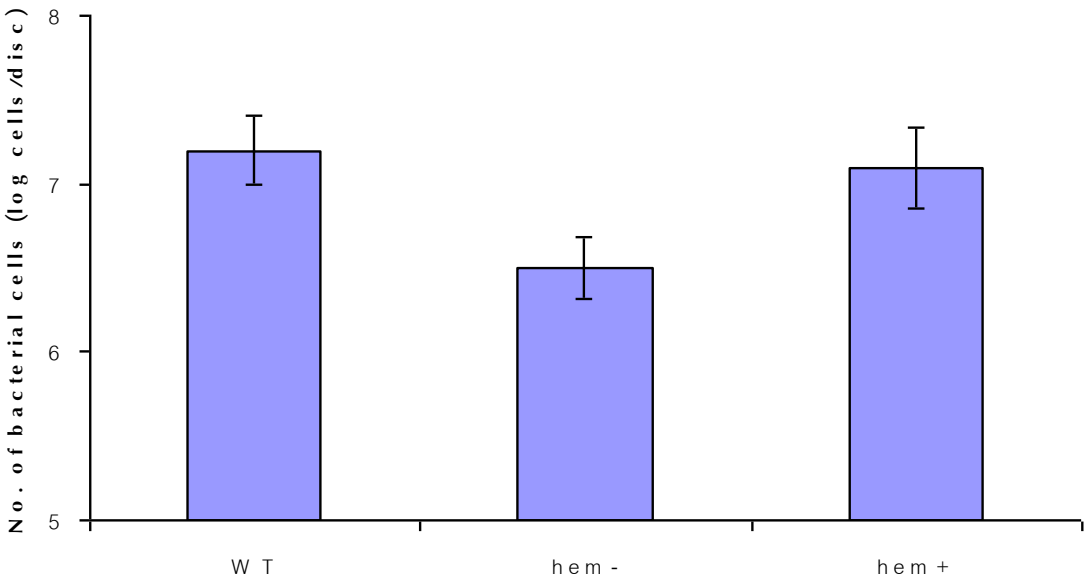

Figure 8. Attachment of the *Xanthomonas axonopodis* pv. *glycines* strains to the surface of soybean leaves after immersing individual leaves into cell suspension of wildtype (WT), *hem* mutant (*hem*$^{-}$) and complemented *hem* strain (*hem*$^{+}$)(10^{7} cells/ml) at 28°C.

of *hem* mutants cells was attachted soybean leaves surface significantly lower than the wildtype and complemented *hem* mutants (Figure 8).

Motility analysis

The *hem* mutant cells were swarming motile on semi-solid medium with 0.4% agar same as the wildtype and complemented *hem* mutant. However, we found that the swimming ability of the *hem* mutants on 0.25% agar swimming plate was significantly reduced when compared with the wildtype and complemented *hem* mutants (Figure 9). This result suggests that the *hem* mutants have effect to swimming motility but not for swarming movement.

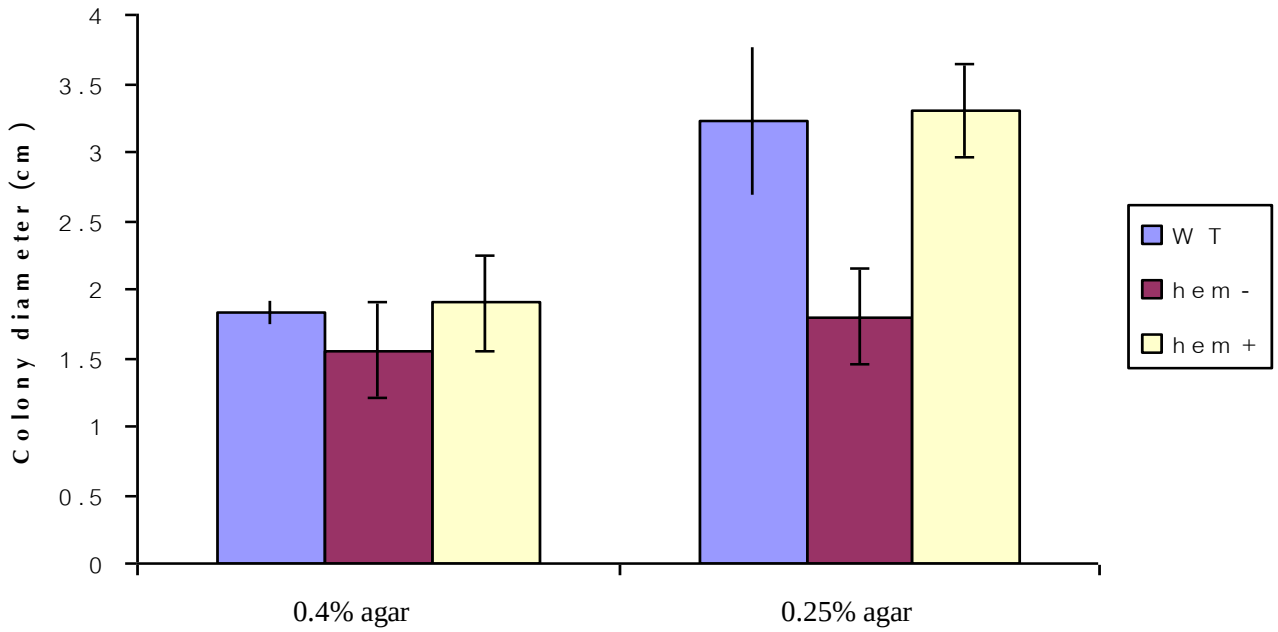

Figure 9. The motility zone of colonies grown in swimming and swarming plates with 0.25% and 0.4% agar respectively was measured at 2 days. WT = wildtype, *hem*$^{-}$ = *hem* mutant and *hem*$^{+}$ = *hem* complementary strain. Each data point is an average of 4 independent experiments, error bars indicate the standard error. The results are representative of four independent experiments.

Extracellular enzyme production

The production of extracellular proteases, amylases, and cellulase have been shown to be virulence factors in *X. axonopodis* pv. *glycines* 12-2 (Thowthampitak et al., 2008). To explore whether the *hem* mutant might also effects such virulence factors, these traits were compared among the wildtype, *hem* mutant, and complemented *hem* mutants in diffusion plate assay. We found that the expression of these extracellular enzyme productions did not differ among them (data not shown).

DISCUSSION

The acquisition of iron is thus one of the most important adaptive responses for bacterial pathogens. The ability of pathogenic bacteria to acquire iron from free heme and host hemoproteins has been studied by many laboratories for clinical pathogens (Braun et al., 1998; Lee and Levesque, 1997) but has not been reported in bacterial plant pathogens. We also found the sequence of hemin uptake locus system in draft genome of a bacterial pustule pathogen *X. axonopodis* pv. *glycines* 12-2 (GenBank accession number AJJO01000000). Sequence analysis of the hemin uptake locus revealed three genes including hemin uptake protein (Xag857), hemin uptake system outer membrane receptor (Xag858), and hemin transport protein (*hem*) required for use of hemin and hemoproteins as iron sources. In this study, we have analyzed the function of *hem* gene coding for hemin transport protein of *X. axonopodis* pv. *glycines* 12-2 that effect to virulence on soybean in the epiphytic phase of infection involving the extracellular polysaccharide (EPS) production, biofilm formation, attachtment, and motility. These might be suggesting that this *hem* system might be important for bacterial-plant interaction.

The colonization as epiphytic of *X. axonopodis* pv. *glycines* before infects through stomata or wounds on soybean leaves is very important process for cause pustule disease on soybean. In this report, the *hem* mutant of *X. axonopodis* pv. *glycines* was virulence deficient and decrease in the population size when sprayed on soybean plants but not when injected directly to soybean leaves. These results suggest that the hemin transport protein is essential in epiphytic phase, but not required for endophytic phase**.** Therefore, the reduction in virulence of the *hem* mutant is possible that *hem* might affect the expression of genes involving in the epiphytic fitness. Previous reports have indicated that *hem* is a multifunctional regulatory protein which controls the expression of trypsin-like protease, hemagglutinating, and hemolysin activities, as well as the production of extracellular vesicles in *Porphyromonas gingivalis* (Carman et al., 1990). Disruption of the hemin transport protein would result in the effected expression of these factors in *X. axonopodis* pv. *glycines*. This hypothesis is consistent with the regulation of hemin-responsive genes

in bacteria by a negative regulator such as the well-described *E. coli* ferric uptake regulator (Fur). Fur acts as a classical negative regulator and uses Fe^{2+} as a core-pressor to bind the promoter region of iron-regulated genes (Bagg and Neilands, 1987). Regulation of iron-regulated genes by a Fur-like system has been found in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* plants (Jittawuttipoka et al., 2010; Subramoni and Sonti, 2005). The mutations in ferric uptake regulator were also resulted in the reduction in virulence of *X. oryzae* pv. *oryzae* and *X. campestris* pv. *campestris* on their host plants (Jittawuttipoka et al., 2010; Subramoni and Sonti, 2005).

In this study, the transcription level of *hem* was reduced when bacteria grow in soybean plant. After invasion into the leaf through stomata, bacteria multiply within the substomatal chambers and intercellular spaces of the spongy mesophyll (Jones and Fett, 1985). In the plant cells, iron concentration was higher than in the surface of the plant. Many iron and heme transport systems are repressed by iron and Fur under iron-rich conditions (Lee, 1995). A component of an iron-scavenging system (PSPTO2134) of *P. syringae* pv. *tomato* DC3000 appears to be repressed under high iron concentration conditions (Jones and Wildermuth, 2011). While virulence control by iron has been well illustrated for the Fur system in *P. aeruginosa*, the Fur system is a negative regulator, indicating that the system represses the uptake of iron when iron is rich (Lamont et al., 2002). Furthermore, expression analyses in *P. syringae* pv. *tomato* DC3000 cultures indicates that high iron [50 μM iron(III) citrate] both represses high-affinity iron-scavenging system expression and induces expression of the type III secretion system and virulence genes in culture (Jones and Wildermuth, 2011).

From our data it seems that *hem* was suppressed in the high iron concentrations at 6 mM and in the plant cells which assumed is an also high iron concentration. Therefore, *hem* mutant shows the disease severity as a wildtype when inject the cells directly to soybean plant. Other pathogens that colonize similar plant environments (that is, the leaf apoplast and vasculature) might be expected to be similarly. The addition of bean leaf apoplastic fluid to *Pseudomonas syringae* pv. *phaseolicola* NPS3121 grown in minimal medium resulted in the expression of virulence genes and the repression of high-affinity iron import systems (Hernández-Morales, 2009). It was expected that abundant iron would repress high-affinity iron scavenging, but the induction of virulence genes was surprising. In a follow-up study, Kim et al. (2009, 2010) found that expression of virulence factors of *Pseudomonas syringae* pv. *tomato* DC3000 in *hrp*-inducing minimal medium was limited by iron availability and that higher iron to well above 10 μM continued to induce higher virulence gene expression. Indeed, we observed that the *hem* mutant did not affect type III genes expression under *hrp*-inducing minimal medium, this result suggesting that type III secretion system may be involved via a mechanism independent of the *hem* gene. For growth in *vitro*, the *hem* mutant exhibits an increase in sensitivity to iron when grown in the high iron media, compared to the wildtype. The growth effect of *hem* mutant under high iron concentration might be due to iron catalyzes the Fenton reaction as in Fur and Zur systems. This growth defect phenotype also found in the *fur* and *zur* mutants in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* respectively (Jittawuttipoka et al., 2010; Tang et al., 2005; Yang et al., 2007). The *zur* mutant was also exhibited an increase in sensitivity to zinc or iron when grown in the high zinc or iron media compared to the wildtype in *X. oryzae* pv. *oryzae* (Yang et al., 2007). In case of *P. syringae* pv. *tomato* DC3000, the iron-rich condition is around 200 μM and iron toxicity begins at over 400 μM. The toxicity was expected because iron catalyzes the Fenton reaction, producing the highly reactive hydroxyl radical, that result in reduced aerobic growth (Andrews et al., 2003). Moreover, the haem-uptake gene cluster in *Vibrio fischeri* is also regulated by Fur and contributes to symbiotic (Septer et al., 2011).

The successful establishment of a pathogen within a specific niche requires the ability of the pathogen to sense the specific environmental conditions of the host and to regulate the expression of virulence genes accordingly (Mekalanos, 1992). It is interesting to speculate that in response to hemin limitation, *X. axonopodis* pv. *glycines* is capable of turning on the expression of several factors which appear to be involved in the virulence potential of this organism. In this study report that *hem* was affected to the EPS production, biofilm formation, attachment, and motility but did not for extracellular enzymes production. This inference is consistent with the observations of hemin uptake system to enhanced expression of several putative virulence factors by *Streptococcus pneumoniae* and *Porphyromonas gingivalis* in mammalian hosts (Tai et al., 1993; Genco et al., 1995). Similar to the *zur* gene, the *X. oryzae* pv. *oryzae zur* mutant decreased the production of EPS and virulence on rice (Yang et al., 2007).

Previous report indicated that the existence of significant amounts of iron in corn seeds affected in bacterial adhesion and host colonization processes (Jacobs and Walker, 1977). The effect of hemin system to a role in EPS synthesis, and reduction in biofilm formation has also been observed in hemin transport protein mutants in *Yersinia* (Jarrett et al., 2004).

Importantly, we also found that the *hem* mutant was deficient in adhesion to both abiotic surfaces and soybean leaf surfaces. A clinical study has shown that iron depletion alters the cell surface property of pathogenic bacteria and lowers their attachment to sur-faces (Harjai et al., 1996). It is therefore possible that iron limi-

tation reduces the ability of the *hem* mutant to attach to soybean leave leading to a lower colonization rate. In contrast with *P. gingivalis*, the decreased transport of hemin by *P. gingivalis* results in the increased expression of hemolytic and trypsin-like protease activities that may contribute to the enhanced invasiveness exhibited in the mouse subcutaneous chamber model (Genco et al., 1995). Moreover, we also found that the swimming ability of the *hem* mutant was reduced. For marine bacteria that specialize in living on particles and aggregates, the population swimming speed of marine bacteria were significantly reduced in no-iron treatment. This reduction in population swimming speeds resulted in lower diffusivity and subsequently a lower colonization rate (Tang and Grossart, 2007).

We were surprised to find that the level of extracellular enzyme productions was not differed in the *hem* mutant compared with the Xag wildtype. Since changes in quorum sensing are associated with varying iron levels and quorum sensing of Xag controls a variety of traits including extracellular enzymes production, EPS production, motility, and biofilm formation that contribute to the virulence and epiphytic fitness (Thowthampitak et al., 2008). In *Xanthomonas campestris* pv. *campestris*, the strain deficient in exbD2, which encodes a component of its unusual elaborate TonB system, had impaired pectate lyase activity and caused no visible symptoms for defense on the non-host plant pepper (Vorhölter et al., 2012). It seems possible that extracellular enzyme productions of Xag may be regulated by other iron uptake pathway. Indeed, disruption of *hem* impairs the epiphytic fitness of Xag, as observed by a significant decrease in the population size of the mutants on soybean leaves compared to wild-type. It might thus be expected that, *hem* of *X. axonopodis* pv. *glycines* strongly effects to the ability of bacteria to EPS production, biofilm formation, swimming motility and thus lead to advantage for survival and colonization on leave surface, dispersal throughout the soybean plant, and start the new cycle of pustule disease.

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Full Length Research Paper

Detection of antibiotic resistant *Enterobacteriaceae* from dogs in North West University (South Africa) animal health hospital

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Bacteria belonging to the family *Enterobacteriaceae* are facultative anaerobic, Gram-negative, non-spore forming rod-shaped bacilli. Members of this heterogeneous group of bacteria do not only form part of the normal flora of humans and animals, but are also widely distributed in various environments such as water, soil and plants. Most members of the *Enterobacteriaceae* were previously considered to be harmless. However, there is evidence that some strains potentially cause diseases and pathological conditions such as diarrhoea, gastroenteritis, urinary tract infections and inflammatory bowel diseases in animals and humans. The aim of the present study was to isolate and determine the antibiotic resistant profiles of *Enterobacteriaceae* isolated from dogs that visited the North West University animal hospital. Fifteen (15) faecal samples were collected from the rectum of dogs that visited the Hospital, using sterile swabs and the samples were placed in transport media. The samples were immediately transported on ice to the laboratory for analysis. MacConkey agar with crystal violet was used for selective isolation of bacteria belonging to the family *Enterobacteriaceae*. Only isolates that satisfied the preliminary identification tests (Gram staining, triple sugar iron agar test, citrate agar test and oxidase test) and confirmatory identification test (API 20E) were retained for further analyses. Antibiotic susceptibility tests were performed on all positively confirmed isolates to determine their antibiotic resistant profiles against tetracycline (30 µg), ampicillin (10 µg), amoxicillin (10 µg), penicillin (10 µg), gentamycin (30 µg) and streptomycin (10 µg). A total of 120 isolates were positively identified as members of the *Enterobacteriaceae*. All the isolates were Gram negative rods and oxidase negative. A large proportion (92.5%) of these isolates fermented the sugars in the TSI agar with only a small proportion (23.3%) producing hydrogen sulphide gas. However, a relatively larger proportion of these isolates (62.5%) produced gas from the fermentation of sugars. On characterizing these isolates for the ability to hydrolyze citrate, a large proportion (71.7%) were negative for this test. The API 20E test results indicated that bacteria species belonging to four main genera (*Escherichia*, *Salmonella*, *Shigella* and *Klebsiella*) were indentified. A large proportion (50%) of these isolates were identified as *Escherichia coli* while 25, 15.8 and 9.2% were *Salmonella* spp., *Klebsiella* spp. and *Shigella* species, respectively. Isolates from all the samples were most often, resistant to penicillin, ampicillin, tetracycline and amoxicillin while very little resistance was observed against gentamycin and streptomycin. The MDR phenotypes PG-AP-A-T, PG-AP-A-T-S, PG-AP-A, PG-A-T and PG-AP-A-T-GM-S were dominant in isolates from samples analyzed. Although a large proportion of the isolates were resistant to three or more antibiotics, a cause for concern was the fact that some isolates were resistant to all antibiotics screened. The identification of multiple antibiotic resistance among the isolates ignites the need to establish appropriate testing procedures before the administration of drugs to animals, thus reducing the possibility of the development and transfer of antibiotic resistant genes between animals and humans.

Key words: *Enterobacteriaceae*, multiple antibiotic resistance, multi drug resistance (MDR) phenotypes.

INTRODUCTION

In developing and developed countries, humans have a strong relationship with pets such as cats and dogs (Robertson et al., 2000). These animals live as companions in households where they contribute to the social, physical and emotional development of children and the well-being of their owners (Jennings, 1997). Companion animals such as dogs and cats are given certain privileges like spending time on the furniture (Wieler et al., 2011). Despite the fact that pets are significantly beneficial to the society, there are a number of health hazards associated with owning a pet (Manian, 2003; Damborg et al., 2009). Moreover, the number of human patients that are highly exposed to these health hazards is on the increase considering the increase in intensive care provided to these companion animals (Bush et al., 2011; Wieler et al., 2011).

Bacteria belonging to the family *Enterobacteriaceae* are facultative anaerobic, Gram negative, non-spore forming rod-shaped bacilli (Ghotaslou et al., 2009; Ateba and Setona, 2011). Within this family are members of the genus *Escherichia, Shigella, Salmonella, Proteus, Yersinia, Klebsiella, Erwinia, Enterobacter, Citrobacter, Providencia, Hafnia, Morganella, Edwardsiella* and *Serratia* (Blood and Curtis, 1995). This heterogeneous group of bacteria does not only form part of the normal flora of humans and animals, but are also widely distributed in various environments such as water, soil and plants (Lima-Bittencourt et al., 2007). The presence of these bacterial species in the gastrointestinal tract of humans and companion animals play an imperative role in maintaining both the normal digestive and immune functions of the hosts (Hall, 2004). In addition, these bacteria species have also been found to participate in metabolic activities that save energy and absorbable nutrients as well as protect the colonized host against invasion by foreign microbes (Guarner, 2006).

Despite the fact that most members of the *Enterobacteriaceae* were previously considered to be harmless, it is evident that some strains potentially cause diseases and pathological conditions such as diarrhoea, gastroenteritis, urinary tract infections and inflammatory bowel diseases in humans, and companion animals (Nakazato et al., 2004; Greiner et al., 2007; Costa et al., 2008; Suchodolski et al., 2010). It is therefore important to determine the occurrence of these bacterial species in companion animals in a country like South Africa where individuals keep them as pets. The NWU hospital provides veterinary health services to companion animals of individuals who live in the Mafikeng area. The aim of the study was to isolate and determine the antibiotic resistant profiles of *Enterobacteriaceae* isolated from dogs that visited the NWU animal hospital.

MATERIALS AND METHODS

Area of the study

This study was conducted in the North West University Mafikeng Campus, North-West Province, South Africa. Fifteen faecal samples were collected from the rectum of dogs that visited the North West University Animal Hospital, using sterile swabs and the samples were placed in transport media. The samples were immediately transported on ice to the laboratory for analysis.

Laboratory analysis

Selective isolation of Enterobacteriaceae

Rectal swabs obtained from animals were washed in 5 ml of 2% peptone water and then homogenized by vortexing. Ten fold serial dilutions were prepared using the homogenized mixture of faecal sample and a sterile peptone. Aliquots of 100 µl from each dilution were spread-plated on MacConkey agar that contains crystal violet for selective isolation of bacteria belonging to the family *Enterobacteriaceae*.

Bacterial identification

Gram staining

All presumptive isolates were subjected to Gram staining reaction using standard methods (Cruikshank et al., 1975). *Enterobacteriaceae* are Gram negative rod-shaped bacteria, hence all isolates that satisfied this criterion were subjected to preliminary biochemical identification tests.

Preliminary biochemical identification tests for Enterobacteriaceae

Triple sugar iron agar test: Triple sugar iron (TSI) agar (Biolab) obtained from Merck, SA, was used to distinguish members of the family *Enterobacteriaceae* from other Gram-negative bacteria based on the ability of the organisms to metabolize the three sugars: glucose; sucrose; and lactose at concentrations of 1, 0.1 and 0.1% respectively (Prescott et al., 2002). The test was performed as previously recommended (United States Pharmacopeial Convention; Inc., 2001). In performing the test, the media was prepared and aliquots of 5 ml were poured in sterile bottles. The media was sterilized and bottles kept in slanting positions in order to obtain a slant and butt when media solidified. All isolates were subjected to the test streaking the isolates on TSI agar slant and also stab inoculating into the butt using a sterile pin. The inoculated bottles were incubated at 37°C for 24 h. After incubation, the isolates were evaluated for the ability to ferment the sugars present with or without the production of acid, gas and hydrogen sulphide (H_2S). Results were recorded and analyzed as previously recommended (Forbes and Weissfeld, 1998).

Oxidase test: The oxidase test was performed using the oxidase test reagent from Pro-Lab Diagnostics- United Kingdom. The oxidase test is based on the principle that tetramethyl-p-phenylenediamine is oxidised by bacterial cytochrome in the presence of atmospheric oxygen to form purple coloured compound. In performing the test, a single colony was placed on Whatman filter paper (Whatman International Ltd, Maidstone, England). A drop of the oxidase test reagents was added on the paper. The two were mixed using sterile wire loop and the results were read within 30 seconds. The results were recorded based on colour change in which the formation of a purple colour was reported as a positive result and vice versa. However, bacteria belonging to the

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Table 1. List of antibiotics used during the study. The concentrations used as well as inhibition zone measurements in (mm) considered resistant (R), intermediate (I), and susceptible (S) are shown according to NCCLS (1999)

Group	Antibiotic	Abbreviation	Disc conc.	R	I	S
Aminoglycosides	Streptomycin	S	10 µg[a]	≤11	12-14	≥15
	Gentamycin	GM	30 µg[b]	≤12	13-16	≥17
Beta- lactams	Ampicillin	AP	10 µg[a]	≤11	12-14	≥15
	Penicillin	PG	10 µg[a]	≤11	12-21	≥22
	Amoxycillin	A	10 µg[a]	≤11	12-21	≥22
Tetracyclines	Tetracycline	T	30 µg[b]	≤14	15-18	≥19

The superscripts [a] and [c] indicate the concentrations of the discs according to the standard method as stipulated by the manufacturer, Mast Diagnostics, Merseyside, United Kingdom.

family *Enterobacteriaceae* are oxidase-negative and the results obtained for all the isolates are shown in the Appendix Tables.

Simmons citrate utilization test: In performing the test, isolates from a pure colony were streaked on the slant and stab inoculated into the butt of Simmons citrate agar (Fluka, Biochemika) using a sterile pin. The inoculated cultures were incubated at 37°C for 24 h. After incubation a colour change from green to blue was recorded as a positive reaction and vice versa (Brenner, 1984).

Confirmatory biochemical tests for *Enterobacteriaceae*

Analytical Profile Index (API 20E)

Presumptive species confirmation was done using the API 20E test. The API 20E is a standardized test kit intended to facilitate the identification of bacteria belonging to the *Enterobacteriaceae*. The test was performed following the manufacturer's protocol (BioMerieux, France). In performing the test, the microtubes were inoculated with bacterial suspensions. After inoculation, the test strips were incubated at 37°C for 24 h. The results were read with or without the addition of reagents. Results were interpreted using the manual provided by the manufacturer and indices generated were used to determine identities of the isolates with the API web software.

Antibiotic susceptibility tests

Antibiotic susceptibility tests were performed on all positively confirmed isolates to determine their antibiotic resistant profiles using the Kirby-Bauer disc diffusion technique (Kirby-Bauer et al., 1966). The antibiotics tested are shown in Table 1 and the test was performed as recommended by National Committee for Clinical Standards (NCCLS, 2000). Bacterial suspensions were prepared using fresh cultures and aliquots of 100 µl from each suspension were spread-plated on Muller-Hinton agar (Merck) plates.

The antibiotic discs were placed on the inoculated plates using a sterile needle and the plates were incubated aerobically at 37°C for 24 h. The isolates were classified as susceptible, intermediate resistant and resistant by measuring the diameter of the zone of inhibition and comparing them with standard reference values (Table 1). Table 1 presents the details of antibiotics used in the study.

RESULTS

The detection of *Enterobacteriaceae* in animal samples

Fifteen faecal samples collected from the rectum of dogs that visited the North West University animal hospital were analyzed for the presence of bacteria species belonging to the family *Enterobacteriaceae*. A summary of the isolates that satisfied both the preliminary and confirmatory identification characteristics for *Enterobacteriaceae* are shown in Table 2. As shown in Table 2, all the isolates were Gram-negative rods and oxidase negative. A large proportion (92.5%) of these isolates fermented the sugars in the TSI agar with only a small proportion (23.3%) producing hydrogen sulphide gas. However, a relatively larger proportion of these isolates (62.5%) produced gas from the fermentation of sugars. On characterizing these isolates for the ability to hydrolyze citrate, a large proportion (71.7%) were negative. The API 20E test results indicated that bacteria species belonging to four main genera (*Escherichia*, *Salmonella*, *Shigella* and *Klebsiella*) were indentified. A large proportion (50%) of these isolates were identified as *Escherichia coli* while 25, 15.8 and 9.2% were *Salmonella* spp., *Klebsiella* spp. and *Shigella* species, respectively.

Percentage antibiotic resistance of *Enterobacteriaceae* isolated

A total of 120 isolates positively identified as members of the *Enterobacteriaceae* were subjected to antibiotic susceptibility tests. The proportion of isolates resistant to a particular antibiotic was determined and results expressed as percentages. Table 3 indicates the percentage

Table 2. Proportion of isolates from different samples that satisfied both preliminary and confirmatory identification characteristics for *Enterobacteriaceae.*

Sample no.	Gram staining		Oxidase		TSI			Citrate Utilization		API 20E
	+ve	-ve	+ve	-ve	Sugar fermentation	H_2S	Gas	+ve	-ve	
DAH1		8		8	8	0	8	1	7	8 (*Escherichia coli*)
DAH2		8		8	8	6	8	3	5	6 (*Salmonella* spp.) 2 (*Escherichia coli*)
DAH3		8		8	8	0	7	1	7	8 (*Escherichia coli*)
DAH4		8		8	8	7	8	4	4	7 (*Salmonella* spp.) 1 (*Klebsiella* spp.)
DAH5		8		8	8	1	8	1	7	1 (*Salmonella* spp.) 2 (*Escherichia coli*) 5 (*Shigella* spp.)
DAH6		8		8	7	0	4	1	7	4 (*Escherichia coli*) 4 (*Klebsiella* spp.)
DAH7		8		8	8	2	3	3	5	1(*Salmonella* spp.) 6 (*Shigella* spp.)
DAH8		8		8	8	0	8	8	0	8 (*Escherichia coli*)
DAH9		8		8	8	0	1	1	7	8 (*Escherichia coli*)
DAH10		8		8	4	0	3	2	6	8 (*Escherichia coli*)
DAH11		8		8	8	4	6	1	7	4 (*Salmonella* spp.) 4 (*Klebsiella* spp.)
DAH12		8		8	8	2	0	2	6	1(*Salmonella* spp.) 6 (*Escherichia coli*)
DAH13		8		8	8	1	4	3	5	1 (*Klebsiella* spp.) 7 (*Escherichia coli*)
DAH14		8		8	8	3	3	1	7	2(*Salmonella* spp.) 4 (*Klebsiella* spp.)
DAH15		8		8	8	2	4	2	6	3 (*Salmonella* spp.) 5 (*Klebsiella* spp.)
Total		120		120	111	28	75	34	86	

+ve=Positive; -ve=negative

of antibiotic resistant profiles of isolates tested. As shown in the table, isolates from all the samples were most often resistant to penicillin, ampicillin, tetracycline and amoxicillin.

However, very little resistance was observed against gentamycin and streptomycin.

MDR phenotypes of *Enterobacteriaceae* isolated

The predominant multiple antibiotic resistant phenotypes of isolates obtained are shown in Table 4. The MAR phenotypes PG-AP-A-T and PG-AP-A-T-S were dominant in isolates from samples 2 (DAH2) and 4 (DAH4) and were obtained at percentages of 62.5% each. Moreover, phenotypes PG-AP-A and PG-A-T were also obtained at 50%, respectively amongst isolates from samples 6 (DAH6) and 8 (DAH8).

Table 3. Percentage of antibiotic resistance of *Enterobacteriaceae* isolated.

Sample No		PG	AP	T	A	GM	S
DAH1	NR	2	3	7	3	2	2
	%R	25	37.5	87.5	37.5	25	25
DAH2	NR	8	5	5	5	0	5
	%R	100	62.5	62.5	62.5	0	62.5
DAH3	NR	8	8	8	8	3	4
	%R	100	100	100	100	37.5	50
DAH4	NR	5	5	8	5	0	0
	%R	62.5	62.5	100	62.5	0	0
DAH5	NR	0	0	8	0	0	0
	%R	0	0	100	0	0	0
DAH6	NR	4	4	1	4	0	1
	%R	50	50	12.5	50	0	12.5
DAH7	NR	8	3	2	3	2	2
	%R	100	37.5	25	37.5	25	25
DAH8	NR	8	4	5	0	0	0
	%R	100	50	62.5	0	0	0
DAH9	NR	7	2	2	3	2	0
	%R	87.5	25	25	37.5	25	0
DAH10	NR	8	2	4	7	0	0
	%R	100	25	50	87.5	0	0
DAH11	NR	2	0	8	2	0	0
	%R	25	0	100	25	0	0
DAH12	NR	7	0	5	0	0	0
	%R	87.5	0	62.5	0	0	0
DAH13	NR	4	3	4	7	1	0
	%R	50	37.5	50	87.5	12.5	0
DAH14	NR	5	2	7	4	1	0
	%R	62.5	25	87.5	50	12.5	0
DAH15	NR	1	2	8	2	2	0
	%R	12.5	25	100	25	25	0

PG (Penicillin), Ap (Ampicillin), A (Amoxycillin), T (Tetracycline), GM (Gentamycin), S (Streptomycin).

Table 4. The predominant MAR phenotypes for *Enterobacteriaceae* isolated.

Sample no.	Phenotype	No observed	Percentage
DAH1	PG-AP-A-T-GM-S	2	25
	PG-AP-AT-GM	1	12.5
DAH2	PG-AP-A-T-S	5	62.5
DAH3	PG-AP-A-T-GM-S	3	37.5
DAH4	PG-AP-A-T	5	62.5
DAH6	PG-AP-A	4	50
DAH7	PG-AP-A-T-GM-S	1	12.5
DAH8	PG-A-T	4	50
DAH9	PG-AP-A	1	12.5

DAH=Dog Animal Health; NT=Number Tested.

The phenotype PG-AP-A-T-GM-S was obtained at 25% and 37.5% from samples 1 (DAH1) and 3 (DAH3), respectively. Although a large proportion of isolates were resistant to three or more antibiotics, a major preoccupation was the fact that some isolates were resistant to all antibiotics screened.

DISCUSSION

The main objective of this study was to selectively isolate bacteria belonging to the family *enterobacteriaceae* from faecal samples obtained from dogs that visited the NWU animal hospital in Mafikeng, North-West Province, South Africa. These isolates may cause gastrointestinal infections in these animals, may be self-limiting in some instances and may progress to more severe forms of complications. Generally, bacteria belonging to four genera (*Escherichia*, *Salmonella*, *Shigella* and *Klebsiella*) were successfully isolated and their identities confirmed using both preliminary and confirmatory tests. These isolates were not identified at strain level. However, they belong to strains that are highly pathogenic to animals and even humans who interact with them. Bacteria that belong to the genera isolated have been found to be easily transmitted from animals to humans.

Another objective of the study was to determine the antibiotic resistance profiles of the isolates against a panel of six antimicrobial agents. The main reason was due to the fact that the animal hospital provides health care services to pets of residents of the Mafikeng area. However, the hospital is not equipped with a microbiology diagnostic unit that isolates and screens microbes for antibiotic resistant determinants. This usually results in prolonged treatment of infections in dogs and cats brought to the hospital.

Recently, companion animals such as dogs and cats live in close contact with their owners than was the case some time ago; they have increasingly gained the status of a family member in some urban households (Blouin, 2008). They spend time on furniture at home or close face-to-fur contact. Due to increasing intensive care

provided to the animals, the human population is also exposed to risks such as the acquisition of antibiotic resistant strains (Hossain et al., 2004; Sidjabat et al., 2006; Umber and Bender, 2009). With this reality, several studies have been carried out to determine the antibiotic resistant profiles of microbes in general and *Enterobacteriaceae* species in particular from companion animals (Walther et al., 2008; Murphy et al., 2009; Umber and Bender, 2009). The increase of antimicrobial resistance in these pathogens is most often accompanied by severe complications in both humans and companion animals (Alekshun and Levy, 2006; Weese, 2008).

The frequencies of resistance to penicillin, ampicillin, amoxicillin and tetracycline were generally high among *Enterobacteriaceae* isolated from dogs. Similar observations had been reported (Sáenz et al., 2001; Costa et al., 2008). Tetracycline and β-lactams are generally used in animal medicine as observed. Moreover, tetracycline is the drug of choice for the treatment of bacterial infection and growth promotion, but its extensive use has contributed to the emergence of resistance (Mulamattathil et al., 2000; Prescott et al., 2002; Threlfall, 2002; Choudhary, 2004; Falsafi et al., 2009). On the contrary, resistance to gentamycin and streptomycin was low for these isolates. These drugs are really used on animals in the clinic.

In conclusion, the identification of multiple antibiotic resistance among the isolates ignites the need to establish appropriate testing procedures. This is motivated from the fact that bacterial that harbour antibiotic resistance determinants can be easily transferred from companion animals and the owners.

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Antimicrobial activity of five plants from Northern Mexico on medically important bacteria

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The aim of this study was to evaluate the potential antimicrobial activity of five medicinal plants from Northern Mexico against ATCC bacteria *Klebsiella pneumoniae* (9183), *Staphylococcus aureus* (BAA44), *Escherichia coli* (O157), *Enterobacter aerogenes* (9180) and *Enterobacter cloacae* (9235) and eight clinical isolated strains (CI) *Klebsiella pneumoniae, Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa, Sarcina lutea* and *Streptococcus pyogenes*. Methanolic extracts of the leaves were tested against these bacterial strains using diffusion on agar method. The extracts showed antimicrobial activity against at least one of the microorganisms tested. *Leucophyllum frutescens* showed antimicrobial activity against *Staphylococcus aureus* (CI) and Escherichia *coli* O157 the minimal inhibitory concentration (MIC) was 28.0 and 30.0 μg/ml, respectively; *Tecoma stans* inhibited the growth of *Staphylococcus aureus* (CI) MIC 36.1 μg/ml; *Fouquieria splendens* showed antimicrobial activity against *Staphylococcus aureus* and *E. coli* O157 MIC 25.0 and 27.1 μg/ml, respectively, *Euphorbia antisyphylitica* resulted active against *E. aerogenes* 9183 and *S. aureus* (CI) MIC 30.1 and 26.8 μg/ml respectively. *Acacia farnesiana* did not show any antimicrobial activity. With the bioassay of *Artemia salina*, only the extract of *L. frutescens* showed toxicity (DL_{50} de 196.7 μg/ml). The dichloromethane soluble fraction of methanolic extract of *L. frutescens* with bioautography assay revealed three bands in the TLC, showed a broad spectrum activity against *S. aureus* (IC). This findings could increase scientific knowledge of medicinal plants from North of Mexico with antibacterial properties.

Key words: Toxicity, antimicrobial activity, methanolic extracts, medicinal plants. bioautography.

INTRODUCTION

Infectious diseases represent an important health problem and represent one of the main causes of morbidity and mortality worldwide, due to the inadequate use of antibiotics and to bacterial resistance (Ortiz et al., 2009). In past years, the problem of bacterial resistance has increased due to the appearance of pathogenic bacteria resistant to antibiotics (Kahkashan et al., 2012). In traditional medicine diverse infectious diseases have been treated with herbal products. Approximately the 80% of the world population have used herbal products to

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Table 1. Plants species from Northern Mexico screened for antimicrobial activity.

Plant (family)	Common Name	Part	Popular use
Tecoma stans (L.) Juss. Ex Kunth (Bignonaceae)	Tronadora	Leaves	Dysentery, diabetes, liver injury.
Acacia farnesiana (L) Willd (Mimosaceae)	Huizache	Flowers and leaves	Dysentery, antispasmodic, antituberculosis.
Euphorbia antisiphylitica (Zucc) (Euphorbiaceae)	Candelilla	Leaves	Purgative, tooth pain, syphilis. infections urinary.
Fouquieria splendens (Engelm) (Fouquieriaceae)	Ocotillo	Leaves	Diuretic, stomach pain, cough.
Leucophyllum frutescens (Berl.) I.M. Johnst (Scrophulariaceae)	Cenizo	Leaves	Dysentery, fever, cough, asthma, liver injury, cataracts.

Sher, 2009; Adame, 2000; Monroy, 2000.

satisfy their primary health care (Shubhi et al., 2010; OMS, 2004). It has been scientifically demonstrated that plants contain secondary metabolites to which biological properties are attributed, and from these properties, drugs have been developed to cure some diseases. Due to this, there is a constant need to find and develop new compounds with antimicrobial potential, and to continue the search of medicinal plants with new mechanisms of action to treat infectious diseases (Egwaikhide et al., 2009).

Mexico has a rich vegetal biodiversity with a long tradition in folk medicine among indigenous communities. These days the interest in traditional medicine is major on treatment of infectious diseases that affect the poorest sector of the population because this practice plays an important role in primary health care (Adame 2000; Wadud et al., 2007). There are various researches focused on the new compounds with biological activity from natural sources. In them, a great number of articles have been directed to antimicrobial activity evaluation on extracts and essential oils from medicinal and aromatic plants. For this, *in vitro* techniques have been used because of its simplicity and reproducibility (Rodriguez et al., 2010).

With this background, the aim of this study was to evaluate the antimicrobial activity *in vitro* of the methanol extracts of five plants *Leucophyllum frutescens* (Berl.) I.M. Johnst, *Acacia farnesiana (L) Willd*, *Tecoma stans* (L.) Juss. ex Kunth, *Euphorbia antisyphilitica* (Zucc), *Fouquieria splendens* Engelm against negative and positive bacteria that cause infectious diseases and reference ATCC strains, determine the toxicity of the extracts with the *Artemia salina* bioassay as well as partially identify the active compounds by assay bioautography. The vegetal species evaluated were selected for their use in traditional medicine to treat tuberculosis, fever, skin diseases gastroenteritis, urinary infectious, gastroenteritis and respiratory infections or conditions related to them (Table 1).

This study will contribute to increase the knowledge of the antimicrobial properties of these five medicinal plants from Northern Mexico on the development of new compounds with potential antimicrobial activitiy.

MATERIAL AND METHODS

Vegetal material

For this investigation, older people and herbalists were interviewed for the selection of the plants with anecdotic evidence for the treatment of infectious diseases on respiratory and gastrointestinal diseases. Fresh plants were recollected during the months of May and July of 2010 from the towns Nazas, Tlahualilo and San Pedro located on Chihuahuan semidesert in Northern Mexico with an altitude of 1200 m above sea level. The plants were authenticated by Eduardo Blanco Contreras of the Universidad Autonoma Agraria Antonio Narro and one sample was kept on the Herbarium in this university.

Extract preparation

The vegetal material was washed with deionized water to eliminate the excess of dust and damaged materials. The leaves were selected as the vegetal species except for *Acacia farnesiana* where the flower was used. The plants were dried in an oven at fixed temperature of 40°C with internal thermometer monitored until dried and ground in a mill.

To obtain the extracts, a mixture of powdered plant material and methanol (CTR Scientific®) in 1:10 proportion was prepared for every plant in an Erlenmeyer flask. The mixture was macerated for five days, and was left in a constant shake. The macerated material was filtered using a vacuum pump. The solvent was eliminated under reduced pressure with a rotating evaporator (Buchii,® R-205, Switzerland) set to 30 rpm and 50° C. The extracts were then completely dried (Rodríguez et al., 2010).

Microorganisms

Five referenced bacteria strains were provided by the Analytical Chemistry Laboratory of the Universidad Autonoma de Nuevo Leon from Monterrey México, and isolate clinical strains were obtained

Table 2. Evaluated microorganism.

ATTC bacterial strain	Clinical isolated bacteria strains (CI)
Klebsiella pneumoniae 9183	*Klebsiella pneumoniae*
Staphylococcus aureus BAA44	*Staphylococcus aureus*
Escherichia coli O157	*Escherichia coli*
Enterobacter aerogenes 9180	*Proteus mirabilis*
Enterobacter cloacae 9235	*Proteus vulgaris*
	Pseudomonas aeruginosa
	Sarcina lutea
	Streptococcus pyogenes

from the Hospital Infantil Universitario from Torreon, Mexico (Table 2).

Identification and conservation of the bacterial strains

The identification and typing of the bacterial strains was realized on biochemical profiles and recommendation of the Clinical Manual of Microbiology (Koneman et al., 2008; Hernández, 2003). All isolates were kept on a liquid medium (Rivas et al., 2007).

Assay microbiological

For the microbiological assay, the crude extracts were screened against eight strains bacterial pathogens (IC) and five reference strains by agar disc diffusion. In this method, 5 ml of culture medium (C. Rivas) was poured in test tubes of 13x100 mm sterilized to 15 lb/15min at 121°C. The tubes were inoculated with each bacterial strain and incubated for 18-24 h at 37°C. With sterile cotton swabs were dipped in the bacterial suspension 100 µl of $1x10^6$ colony forming units (CFU) adjusted with the Mc Farland Nephelometer (Clinical Laboratory Institute, 2006) and evenly streaked over the entire surface of the agar plate to obtain uniform inoculums (Khan et al., 2010).

Crude extracts 50 µL was poured on a Whatman No. 1 (Whatman® International LTD England) paper disc to 1,000, 500 and 250 µg/mL concentration and sterilized by filtration with 0.25 µm Millipore® membranes, over the solid agar. C Rivas was used as positive control 50 µL Cefotaxime (Sigma Aldrich®, St Louis MO, USA) and 50 µL Dimetil sulfóxido was used as the negative control. Each assay was analyzed in triplicate. All the plates were incubated at 37°C for 24 h after this time, the diameter inhibition halo was measured (NCCLS, 2001).

Determination of minimum inhibitory concentration (MIC)

The MIC assay was performed by the microdilution method; the medium used was C. Rivas broth. In 96-well microplate, 100 µL of C. Rivas broth was deposited in each well, and was added to the extract at 500, 250, 125, 62.5, 31.25, 15.6, 7.8 and 3.9 µg/mL concentration, followed by 100 µL of bacterial suspension containing the inoculum $1x10^6$ CFU adjusted with the Mc Farland Nephelometer. Next the microplate was incubated for 24 h to 37°C; for each trial, 500 µg/mL cephotaxime was used as positive control. All assays were performed in triplicate. To determine the MIC extract, 10 µL of indicator solution p-iodine tetrazolium at a concentration of 2.5 mg/mL was added to each well. The microplate was incubated for 8 h at 37°C; absorbance was read in a microplate reader (Dynatech® MR500) at 570 nm. (Yasunaka et al., 2005; NCCLS, 2002; Umeh et al., 2005: Vega et al*.,* 2013).

Bioautography

Evaluation of active chemical compounds was performed by bioautography using *Leucophyllum frutescens* against *Staphylococcus aureus* (IC). The assay was performed by agar overlay bioautography technique. Plant extract sample (5 µl) was applied 2.5 cm from the base of the silica plate (60W Merck)®. After drying, the plates were developed using solvent Benceno-Acetona (8:2), after which chromatography thin layer (TLC) plates were carefully dried for complete removal of solvents. Bioautography was performed with a culture of *S. aureus* (IC) which showed a better antibacterial sensitivity to the dichloromethane fraction extract of *Leucophyllum frutescens.* Aliquot of 20 mL of C. Rivas agar was overlaid on dried TLC plate under aseptic condition in laminar airflow by adding 200 µL of bacterial inoculum (1×10^6 CFU). The TLC plate were incubated at 37°C and examined for the zone of inhibition (Ncube et al., 2008, Schmourlo et al., 2004). Figure 1 shows the three active fractions of *L. frutescens* and inhibition on *S. aureus* isolate (IC).

Toxicity test with *Artemia salina* nauplii.

For the toxicity test, 0.1 g eggs of *Artemia salina* (Brine Shrimp Eggs® San Francisco Bay Brand, INC) were incubated on artificial sea water on a dark container divided by a middle wall with a space of 2 mm in the bottom. To prepare the sea water, 40 g of sea salt (Instant Ocean®, Acuarium System) were weighed with 0.006 g of yeast (Mead Johnson®) after which one liter of bidistilled water was added.

The pH was adjusted to 7.8, the containers were kept in condition of artificial white light and oxygenation, 48 h later, the hatched larvae called nauplii were taken with a Pasteur pipette and transferred to another container and kept in conditions of light, oxygen and temperature 22-29°C for 24 h. On a microplate of 96 wells was placed 100 µl of seawater containing 10 nauplii per well plus 100 µL of vegetal extract having concentrations of 10. 50, 250, 500 and 1,000 µg/mL a four replicates. Was used as positive control potassium dichromate a 400 ppm concentration and sea water was used as negative control. After 24 h with the help of a stereoscopic microscope, the total count of live and dead nauplii per dose was done. Probit method was used to determine the LD_{50} (Bastos et al., 2009, Déciga et al*.,* 2007).

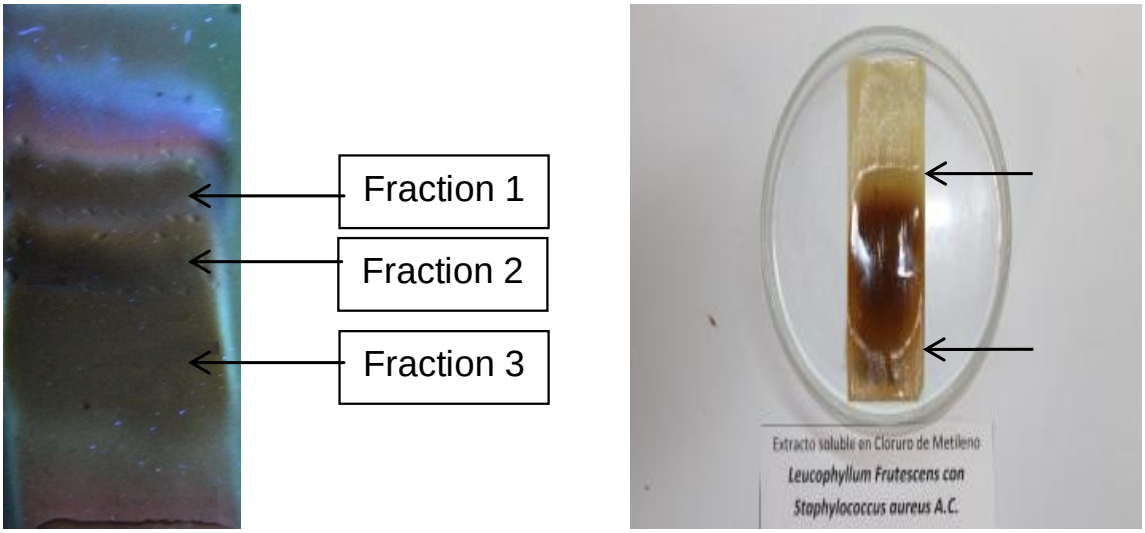

Figure 1. Dichloromethane fraction of *L. frutescens* and inhibition on *S. aureus* (IC).

Table 3. Biological activity of methanolic extracts of vegetal species on study from Northern Mexico against reference bacterial strains ATCC with method agar disc diffusion.

Plant	Microorganism				
	***K. pneumoniae* No 9183**	***S. aureus* No BAA44**	***E. coli* O157**	***E. aerogenes* 9180**	***E. cloacae* 9235**
L. frutescens	+	+ + +	+ +	-	+
F. splendens	-	-	+ +	-	-
E. antisiphylitica	-	-	+ +	+ +	-
A. farnesiana	-	-	-	-	-
T. stans	-	+ +	-	-	-

Scale: 5-10 mm weakly active (+), moderately active 11-15 mm (++), highly active > 15 mm (+++) at the discretion of García et al., 2006.

RESULTS AND DISCUSSION

Plants evaluated were selected by their use on folk medicine and by the few scientific reports on the antimicrobial activity of the flora from North of Mexico. All plant species showed antibacterial activity against tested microorganisms (Tables 3 and 4). Extracts of *L. frutescens, T. stans*, *F. splendens* and *E. antisiphylitica* showed antibacterial activity at concentrations of 250 - 1000 μg/ml against *S. aureus* (IC). In studies performed by Molina-Salinas et al. (2007), methanol leaves extracts of *L. frutescens* were reported to possess antibacterial activity which inhibited the growth of multirresistant strains of *M. tuberculosis*, *S. aureus* and *H. influenzae* b type. Extracts of *L. frutescens* and *T. stans* inhibited the growth of *E. coli* O157 at the concentrations tested. *E. antisiphylitica* showed antibacterial activity against *E. aerogenes* (9183), *F. splendens* inhibited the growth of *K. pneumoniae* (IC). The antibacterial activity showed a linear range with the extracts concentrations. The methanolic flowers extract of *A. farnesiana* showed no significant activity with the bacteria tested. Zaidan et al. (2005) evaluated the methanol leaves extracts of *Morinda citrifolia*, *Piper sarmentosum, Vitex negundo, Andrographis paniculata* and *Centella asiatica* found a high antibacterial activity against *S. aureus* and *S. aureus* methiciline resistant, but none of the five plants studied showed antibacterial activity against Gram negative bacteria; these findings are similar to our study, but different plants.

Other studies with native plants from Northern Mexico desert performed by Cespedes et al. (2006) found that *T. lucida* extracts ($MeOH/CH_2Cl_2$) inhibited the growth of *E. coli, P. mirabilis, K. pneumoniae, Salmonella* spp. and

Table 4. Biological activity of methanolic extracts of vegetal species on study from Northern Mexico against bacterial strains (IC) with method agar disc diffusion.

Plant	Microorganism				
	K. pneumoniae	*S. aureus*	*E. coli*	*E. aerogenes*	*E. cloacae*
L. frutescens	+	+ + +	+ +	-	+
F. splendens	-	++	+ +	-	-
E. antisiphylitica	-	++	+ +	+ +	-
A. farnesiana	-	-	-	-	-
T. stans	-	+ +	-	-	-

Scale: 5-10 mm weakly active (+), moderately active 11-15 mm(++), highly active > 15 mm (+++) at the discretion of García et al., 2006

Table 5. Minimum inhibitory concentration of methanolic extracts of vegetal species on study.

Plant species	Minimum concentration inhibition μg/Ml			
	***E. aerogenes* ATCC 9183**	***E. cloacae* ATCC 9235**	***S. aureus* (IC)**	***E. coli* ATCC O157**
L. frutescens			25.4	30.0
T. stans			36.1	
F. splendens			25.0	27.1
E. antishypilitica	30.1		26.8	

ATCC, American type culture collection; IC, isolate clinical.

Shigella spp. unlike the results of our study; where, Gram negative bacteria showed no activity. Adelou et al. (2009) reported the antibacterial activity of methanol extracts of leaves and stems of *Buddleja saligna* against Gram positive bacteria *S. aureus* and *S. epidermidis*, where they found inhibition of these bacteria, unlike the Gram negative strains of our study. This suggest that in our study the Gram negative bacteria were most resistant than the Gram positive bacteria. *A. farnesiana* did not show activity with bacteria tested. Ruiz et al. (2009) evaluated the antimicrobial and antifungal activity of methanol extract of six medicinal Mexican plants; *Amphypteringium adstrigens, Castella tortuosa, Coutarea latiflora, Ibervillea sonorae, Jatropha cuneata, Selaginella lepidophylla*, plant species different used to in our study, reported that *S. aureus* bacteria was more susceptible to all that plants tested.

The minimum inhibitory concentrations (MICs) for the methanolic extract of *L. frutescens, T. stans, E. antisiphylitica and F. splendens* are shown in Table 5. Our results show that all tested strains bacteria was less or equal to 30 μg/ml, but *S. aureus* show better inhibitory effect. do Nascimento et al. (2013) reported results similar with plant Pimenta malagueta (*Capsicum frutescens*). Bioautography assay of the dichloromethane soluble fraction obtained of methanol leaves extract of *L. frutescens* revealed three bands with chromatography thin layer at final concentration of 1000 μg/mL (Figure 1) which were active against *S. aureus* with halo inhibition of 24 mm. In the results obtained, the fraction were compared with the standard antibiotic used in this study with halo inhibition of 30 mm. Taiwo et al. (2013) reported activity against *S. aureus* concentration of 20 mg/mL with an inhibition even 27 mm with the fraction dichloromethane de *Cassia occidentalis* linn.

The chemical compounds identified in all three bands were two flavonoids and a quinone (Table 6). Mehrotra et al. (2010) studied five plant extracts for evaluating the bioactive components by bioautography, they reported that the extract *Zyzygium aromaticum* (clove) showed at least two active components against *S. aureus*. Bastos et al. (2009) conducted a study with the chloroform extract of *Zeyheria tuberculosa* (Vell.) Bur, found four flavones which were evaluated by bioautography against *S. aureus* and reported that two compounds were active against *S. aureus*.

Based on the fact that the objective was to evaluate the biological activity of extracts of plants, including toxicity, the LD_{50} was determined with the assay of *Artemia salina* lethality, screening test considered biological systems (Lagarto-Parra et al., 2001; Bastos et al., 2009) thereby ensuring their effectiveness and no toxicity. In this paper, we found a LD_{50}> 1,000 mg / mL for the methanol extract of the plants under study, except that *L. frutesens* showed an LD_{50} of 196.7 μg/mL. This result indicates that the extract tends to be toxic in agreement to the study of

Table 6. Rf of three chromatography bands of dichloromethane soluble fraction developed with UV light y $CoCl_2$ with eluent Benceno-Acetona (8:2) and chemical compounds.

Fraction	Rf	Light UV	Cobalt chloride	Chemical compounds
1	0.4	Brown	Brown	Flavonoides
2	0.5	Brown	Brown	Quinonas
3	0.6	Brown	Brown	Flavonoides

Déciga et al. (2010). In a study by Morales (2006) LD_{50} of 64.57 µg/mL of the methanol extract of *Lophocereus schottii* was obtained a on the lethality of crustacean *A. saline*.

Conclusions

The methanolic extracts of five plants native from Northern Mexico: *L. frutescens, T. stans, F. splendens, E. antisiphylitica* and *A. farnesiana* were evaluated against pathogen bacteria of clinical isolates and reference strains. *L. frutescens, T. stans, F. splendens, E. antisiphylitica* extracts showed significant activity over *Staphylococcus aureus* (IC) with MIC 25.4, 36.1, 25.0, 26.8 µg/ml, respectively. The extract that showed higher activity was *L. frutescens* (CMI) 25.4 µg / mL against *S. aureus*. The dichloromethane fraction of *L. frutescens* with Rf 0.4, 0.5 and 0.6 showed activity on *S. aureus* by bioautography. The tested extracts of the five study plants in *A. salina* nauplii showed no toxicity except for *L. frutecens* with a LD_{50} of 196.7µg/mL.

In this study, we obtained results of the antimicrobial activity of the five native species from Northern Mexico, contributing to increasing the knowledge of the plant used to in traditional medicine and could be the basis for further studies to isolate the active compounds of the studied plants and evaluate their effectiveness against other microorganisms.

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Full Length Research Paper

Efficient cellulase production from low-cost substrates by *Trichoderma reesei* and its application on the enzymatic hydrolysis of corncob

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In this work, cellulase production by *Trichoderma reesei* CH11 using different low-cost raw materials as carbon sources and its application on the enzymatic hydrolysis of corncob were systematically investigated. Among all the studied carbon sources, rice straw was the most suitable one for *T. reesei* to produce cellulolytic enzymes. In addition, adding wheat bran and ammonium sulfate improved the cellulase activity and reduced the production cost. *T. reesei* was cultivated at 28°C and 160 rpm for five days to generate the liquid enzyme for hydrolysis. The enzyme activity of FPase reached 2.40 IU/mL, which was improved about 21% compared with that obtained from original culture medium without wheat bran or ammonium sulfate. Pretreated corncob was then hydrolyzed by this crude enzyme. After optimization of solid-to-liquid ratio and dilution rate of crude enzyme, 95.7% of hydrolysis yield was obtained. This work could offer one promising low-cost platform for bio-refinery.

Key words: *Trichoderma reesei*, cellulase, low-cost substrate, corncob, enzymatic hydrolysis.

INTRODUCTION

Recently, the concept of bio-refinery was a hot topic since this potential technology could help in solving the energy crisis or environmental problem (FitzPatrick et al., 2010). Lignocellulosic biomass, the most common substrate for bio-refinery, could be considered as the next generation of energy source (Kumar et al., 2008).

However, lignocellulosic biomass cannot be utilized by most microorganisms for fermentation directly. Thus, hydrolysis is usually necessary for the utilization of lignocellulosic biomass and thus it is the key factor that determines the possibility and profits of bio-refinery (Wyman et al., 2005). Among the two main kind of hydrolysis (chemical and enzymatic one), enzymatic hydrolysis was more efficient and "green" for environment, and thus was the focus for many works (Rubin, 2008). However, the cost of commercial cellulase was too expensive for industrial application (Lynd et al., 2008). Unfortunately, little work focused on the low-cost production of cellulase for enzymatic hydrolysis.

To reduce cost of cellulase production, using low-cost fermentation substrates could be a promising strategy. And among various microorganisms, *Trichoderma reesei* was shown to be an ideal one for cellulases production (Martinez et al., 2008). In this work, the efficient

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#Both authors have the same contribution and are co-first authors.

production of cellulases by *T. reesei* strains using ramie powder, bamboo, rice-straw, corn-straw, corncob and cotton stem as the inexpensive carbon source, respectively, and the characteristics of the produced cellulases were investigated. In addition, the medium for cellulases production by adding wheat bran and ammo-nium sulfate to reduce the cost was further evaluated. The produced cellulases were used to hydrolyze the pretreated corncob. At the same time, the effect of solid-to-liquid ratio and dilution rate of liquid enzyme on the enzymatic hydrolysis were also evaluated. This work can offer one low-cost platform for bio-refinery and is beneficial for its future industrialization.

MATERIALS AND METHODS

Microorganism for cellulase production

T. reesei CH11 (stored by the Laboratory of Energy and Biochemical Engineering, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences) was used for cellulases production. It was maintained on potato dextrose agar (PDA) and stored at 4°C until use.

Lignocellulosic biomass and its pretreatment

In this work, bamboo and ramie were obtained from southern China while rice straw, corn straw, corncob and cotton stem were collected from Liaoning Province of China. All these lignocellulosic biomasses were air dried and crushed into particles with diameter less than 2 mm, and they were used as carbon source directly without any pretreatment.

Pretreated corncob was offered by ZHONGKE New Energy Co., LTD (Ying-Kou, China) and used as the raw material for enzymatic hydrolysis in this work. According to ZHONGKE New Energy Co., LTD, the pretreatment method was the modification of the previous work (Teramoto et al., 2008) that used organic solvents to treat the lignocellulosic biomass. The composition of the pretreated corncob was (% w/w): cellulose 57%, hemicellulose 31%, lignin 7%, and others 5%.

Medium

The medium for seed culture contained 1.0% (w/v) glucose, 0.1% (w/v) peptone, 0.05% (w/v) citric acid, 2% (v/v) Vogel's Medium N (Vogel, 1964), and 0.015% (v/v) Tween 80. The initial pH of the medium was 5.0 to 5.5.

The medium for induction and production of enzyme contained 0.1% (w/v) glucose, 0.1% (w/v) peptone, 0.05% (w/v) citric acid, 2% (v/v) Vogel's Medium N, and 0.015% (v/v) Tween 80. Otherwise to culture *T. reesei*, 2%(w/v) ramie powder, 2% (w/v) bamboo, 2% (w/v) rice-straw, 2% (w/v) corn-straw, 2% (w/v) corncob and 2% (w/v) cotton stem as different carbon source were added into the culture media, respectively. The media was then autoclaved at 121°C for 20 min.

Cellulase production by *T. reesei*

Spores of *T. reesei* were incubated into 500 mL flak containing 200 mL pre-cultural medium metioned above and shaken at 30°C and 200 rpm for 36 h to prepare seed cultures. Then, 10% (v/v) seed cultures were translated into flask containing 100 mL fermentation medium. Cultivation was performed in a rotary shaker at 28°C and 160 rpm for five days. After fermentation, the fermentation broth was centrifuged at 4000 rpm for 10 min and the clear supernatant stored at 4°C was used as liquid enzyme for hydrolysis later.

Determination of enzyme activity

The enzyme activity was determined by using filter paper as reaction substrates. A solution (1 mL) of 50 mg filter paper, 800 µL of 0.1 M citric acid buffer (pH5.0) and 200 µL enzyme liquid were mixed and incubated at 50°C for 30 min (Ghose, 1987). To determine CMCase activity, 800 µL of 1% CMC-Na (in 0.1 M citric acid buffer, pH 5.0) and 200 µL liquid enzyme were mixed and then incubated at 50°C for 30 min (Ghose, 1987). To determine *β*-glucosidase activity, 800 µL of 1% salicylic acid (in 0.1 M citric acid buffer, pH 5.0) and 200 µL liquid enzyme were mixed and then incubated at 50°C for 30 min (Ghose and Bisaria, 1979).

After hydrolysis, the mixture was boiled for 5 min to terminate the reaction. The residual sugars concentration was determined by DNS methods (Miller, 1959). The FPase, CMCase or *β*-glucosidase activity was defined as the amount of cellulase required to produce one µmol reducing sugars in one minute. All the measurement was performed at least twice and data were expressed as the average.

Enzymatic hydrolysis by the enzyme produced by *T. reesei*

Enzymatic hydrolysis of pretreated corncob was performed in 250 ml conical flasks, containing a 100 mL liquid enzyme and 10 g corncob. The initial pH of enzymatic hydrolysis was kept natural and the hydrolysis temperature was 50°C. The hydrolysis was carried out for 72 h without specification.

Sugar concentration analysis by HPLC

Concentration of various sugars (D-glucose, D-xylose, D-cellobiose, etc.) in the corncob hydrolysate were analyzed by HPLC (Waters 2685 systems, Waters Corp., USA), with a RI detector (Waters 2414), and on Shodex Sugar SH-1011 column using 0.5 M H_2SO_4 solution at a flow rate of 0.5 ml/min at 50 °C.

The yield of enzymatic hydrolysis was defined according to previous work (Chen et al., 2007), that is:

Hydrolysis yield (%) = reducing sugar×0.9×100/ polysaccharide in substrate.

RESULTS AND DISCUSSION

Cellulase production by *T. reesei*

As mentioned above, to fulfill effective and efficient enzymatic hydrolysis for bio-refinery, suitable cellulase is important (Himmel et al., 2007). Two aspects should be considered, one is the fermentation cost for cellulase production, and undoubtedly low-cost substrate is beneficial for reducing its cost. On the other hand, the cellulase activity should not be influenced by the low-cost substrate used. Base on both points, the effect of various fermentation substrates including carbon sources and nitrogen sources on cellulase production by *T. reesei* was evaluated.

Table 1. Cellulase production by *T. reesei* using different carbon sources in culture medium.

Carbon source (2%, w/v)	Enzyme activity (IU/mL)	
	FPase	CMCase
Bamboo	0.38	0.87
Ramie powder	1.67	1.27
Rice-straw	1.97	1.34
Corn-straw	1.39	1.25
Corncob	1.78	1.22
Cotton stem	1.42	0.88

Carbon sources for cellulase production

Carbon sources are the most important nutrient materials for fermentation and take a great part in the cost of fermentation substrate. Thus, an effective and low-cost carbon source could undoubtedly reduce the fermentation cost significantly. Compared with other low-cost raw materials, lignocellulosic biomass could be considered a promising one for its great availability in nature and renewable characteristics (Rubin, 2008). Six kinds of lignocellulosic biomass as the carbon sources for cellulase production by *T. reesei* were evaluated in this work, namely, bamboo, ramie powder, rice straw, corn-straw, corncob and cotton stem, respectively.

The enzyme activity of cellulase generated by *T. reesei* on the medium containing the above carbon sources was evaluated (Table 1). The highest cellulase activity was obtained on the medium containing rice straw; PFase and CMCase activity reached 1.97 and 1.34 IU/mL, respectively. Thus, rice straw was the most suitable one for cellulase production by *T. reesei* among these six carbon sources, which was in good accordance to previous works (Karimi et al., 2006; Kim and Dale, 2004).

Rice straw is one common agricultural residue in South China. Also, it is cultivated widely among the tropic and semi-tropic area throughout the word. More than 50 countries generate at least 100,000 tons of rice annually (Kadam et al., 2000). It is also worth noting that the price of rice straw is not expensive. For example, in China, its average price was merely about 300 RMB/ton. Thus, rice straw could be one promising feedstock for industrial production of cellulase for bio-refinery field.

Additive nutrient for cellulase production

Besides carbon sources, adding some suitable additive nutrients could also help in increasing the cellulase activity after fermentation (Wen et al., 2005). In this work, to further improve the cellulase activity, wheat bran was chosen as additive nutrient for cellulase production. Bran is the main byproduct of wheat processing and it is rich in carbohydrates, protein, vitamins and minerals, which is more suitable for *T. reesei* growth (Brijwani et al., 2010; Hassan and Bullerman, 2009). Different wheat bran concentration (0 to 3.0 % w/v) was added into fermentation medium and its effect on the cellulase activity was measured. As shown in Figure 1, additional wheat bran was actually useful to improve the cellulase activity. The highest CMCase and FPase activity was got (1.09 and 2.48 IU/mL respectively) when the wheat bran concentration was 1.0% (w/v). However, further increase of the wheat bran did not contribute to further increase in cellulase activity. Thus, the optimal wheat bran concentration for cellulase production was 1.0%.

Nitrogen sources for cellulase production

Initially, the organic nitrogen peptone was used as nitrogen source for cellulase production by *T. reesei* since it grows fast on the medium containing organic nitrogen source (Ryu and Mandels, 1980). In order to reduce the cost of nitrogen source, inorganic nitrogen ammonium sulfide was used instead of peptone, and the effect of its concentration was also evaluated (Figure 2). When the concentration was lower than 2 g/L, the enzyme activity of cellulase increased as the concentration of ammonium sulfide was higher. However, when its concentration continued to increase, both CMCase and FPase activity decreased instead. It is possible that higher ammonium sulfide concentration would inhibit the microbial growth of *T. reesei*. Overall, the highest cellulase activity was got when the ammonium sulfide concentration was 2 g/L. Obviously, ammonium sulfide could replace expensive peptone for cellulase production.

Calculation on the fermentation cost for cellulase production

Base on the experiment above, three low-cost nutrients including carbon and nitrogen sources were used. To reduce the cost of cellulase production, ammonium sulfate was used to take the place of peptone. Besides, wheat bran and other specific components [0.1% (w/v) glucose, 0.2% (w/v) ammonium sulfate, 0.05% (w/v) citric acid, 2% (v/v) Vogel's Medium N, 0.015% (v/v), Tween 80, 1% (w/v) wheat bran, 2% (w/v) rice straw] were also added into the medium. In China, the price of ammonium sulfate, rice straw, wheat bran, is about 1000, 300, and 1200 RMB/ton, respectively. Thus, the substrate used for cellulase production in this work was low-cost and suitable for bigger application.

When the flasks containing 100 mL of enzyme production medium were inoculated (10% (v/v) inoculum) and cultivated in shaker at 28°C and 160 rpm for 5 days, interestingly, the FPase activity could reach 2.40 IU/mL, and the activity improved about 21% when compared

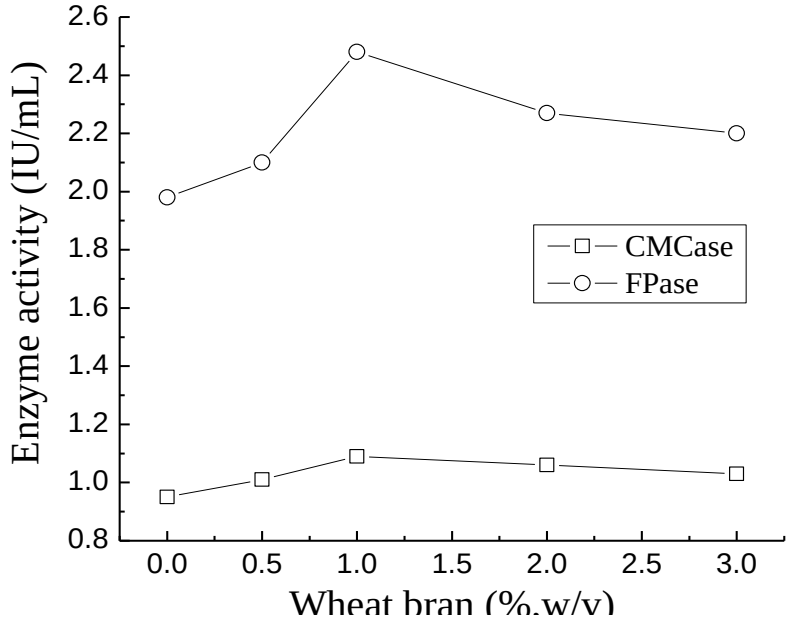

Figure 1. The influence of different concentrations of wheat bran. (□) CMCase; (○) FPase. Xiong et al. (2012).

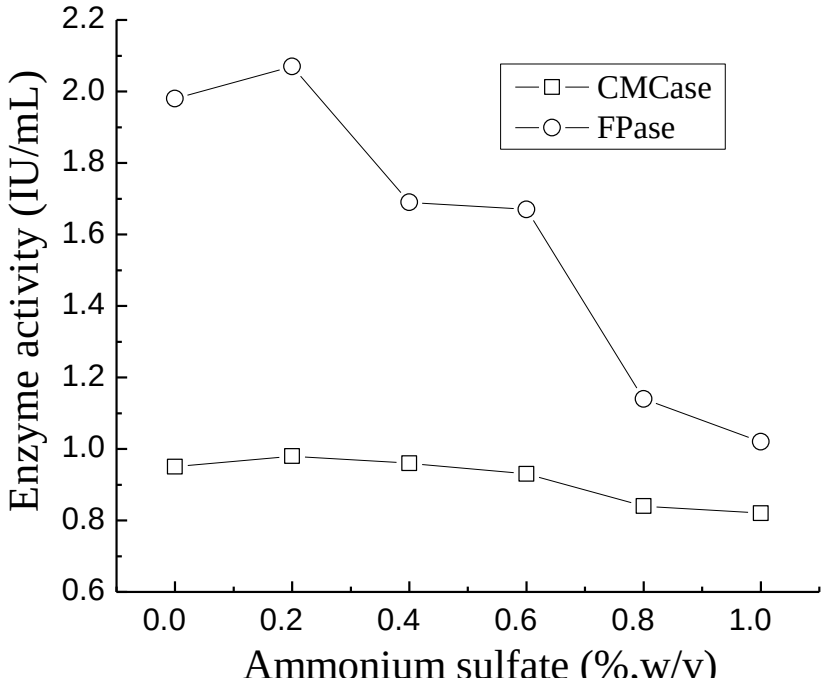

Figure 2. The influence of different concentrations of Ammonium sulfate. (□) CMCase; (○) FPase: Xiong et al. (2012).

with original culture medium without wheat bran or ammonium sulfate. Thus, not only the cost is low for application, using this low-cost fermentation system but was also beneficial for the hydrolysis itself.

Overall, the cost of the substrates mentioned above is low and thus could be ideal feedstock for cellulase

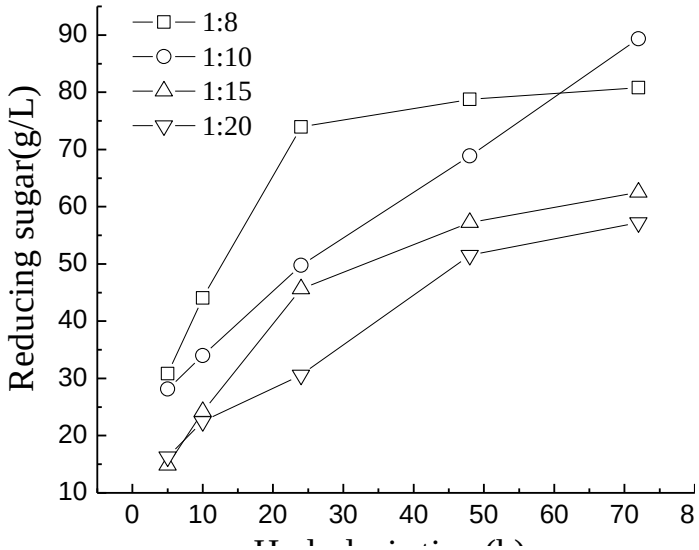

Figure 3. The influence of different solid-to-liquid ratio. □, 1:8; ○, 1:10; △, 1:15; ▽, 1:20: Xiong et al. (2012).

production.

Enzymatic hydrolysis of corncob by cellulase produced by *T. reesei*

In northern and middle China, corncob is one abundant lignocellulosic material that could be used for bio-refinery industrialization (Yinbo et al., 2006). In this work, pretreated corncob kindly offered by ZHONGKE New Energy Co., LTD (Ying-kou, China) was used as the model material for enzymatic hydrolysis by cellulase produced by *T. reesei*.

Effect of solid-to-liquid ratio

Traditionally, solid-to-liquid ratio was one important factor that influences the efficiency of enzymatic hydrolysis. Too low solid-to-liquid ratio might result in low sugar concentration of corncob enzymatic hydrolysate which is not suitable for fermentation and industrialization. Thus, it is critical to increase the solid-to-liquid ratio to get high hydrolysis efficiency (Roche et al., 2009). However, too high solid-to-liquid ratio would bring substrate inhibition and influence the mass transfer during hydrolysis.

To get the optimal solid-to-liquid ratio, its effect on the hydrolysis yield was evaluated. As shown in Figure 3, during the first 24 h of hydrolysis, the fastest hydrolysis rate was at the system with solid-to-liquid ratio of 1:8. At this system, the reducing sugars concentration was 73.9 g/L after 24 h of hydrolysis. However, after that, its hydrolysis rate became much slower. It is possible that too high solid-to-liquid ratio brought substrate inhibition to the enzymatic hydrolysis and made the hydrolysis rate lower. In contrast, at the system with solid-to-liquid ratio of 1:10, the hydrolysis rate was influenced little by the hydrolysis time. After 72 h of hydrolysis, the reducing sugars concentration in this system could reach 89.4 g/L. At the system with solid-to-liquid ratio of 1:15 and 1:20, the reducing sugar concentration was merely 62.5 g/L and 57.2 g/L, respectively after 72 h of hydrolysis.

Effect of diluted rate of liquid enzyme

As mentioned above, the low-cost liquid enzyme produced by *T. reesei* was used for enzymatic hydrolysis. When the hydrolysis was carried out, the liquid enzyme was mixed with the hydrolysis system. For different fermentation, the reducing sugars required in the fermentation medium were different (Cheng et al., 2008; Huang et al., 2009, 2012).

In this work, we simply added the liquid enzyme into the hydrolysis system. Thus, the amount of liquid enzyme added into the system might influence the hydrolysis efficiency. The hydrolysis system was designed as follows: (1) 10 g corncob, 100 mL crude liquid enzyme; (2) 10 g corncob, 50ml enzyme liquid, 50 mL water; (3) 10g corncob, 25 mL enzyme liquid, 75mL water. Surprisingly, we found that tap water was more suitable for enzymatic hydrolysis than pure water.

Also, it is not necessary to adjust the initial pH of tap water. The enzymatic hydrolysis was carried out at 50°C and 150 rpm. As shown in Figure 4, using crude liquid enzyme without dilution for the hydrolysis of corncob, the

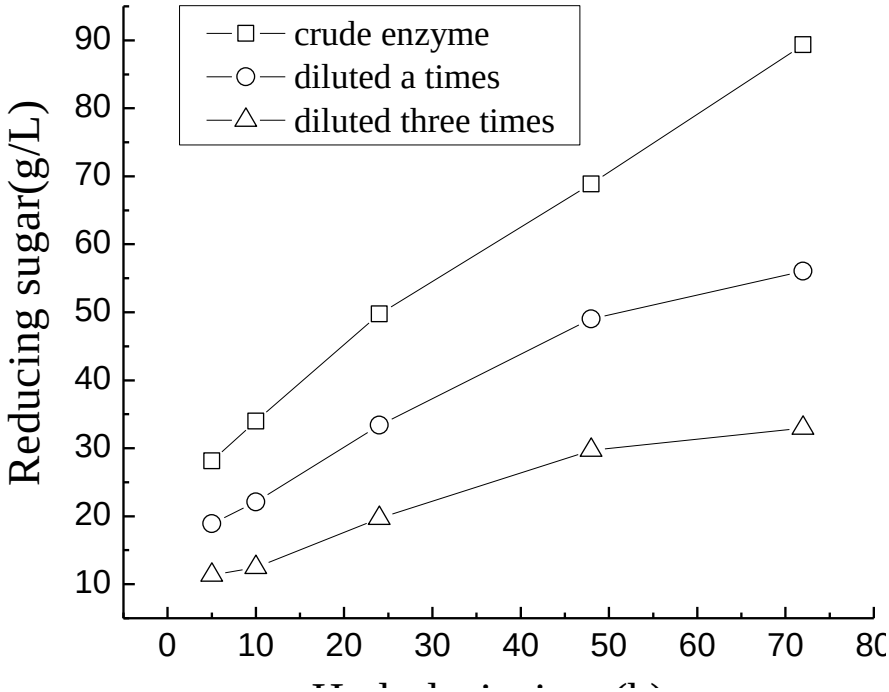

Figure 4. The influence of different diluted times. □, crude enzyme; ○, diluted a times; △, diluted three times: Xiong et al. (2012).

Table 2. Sugars in the hydrolysis system with a solid-liquid ratio of 1:10.

Sugars in hydrolysate	Concentration (g/L)	Hydrolysis yield (%)
Cellbiose	7.8	
Glucose	65.9	95.7
Xylose	20.0	

generation of reducing sugars was faster. After 72 h of hydrolysis, 56.0 and 33.0 g/L reducing sugars were obtained on the system with liquid enzyme diluted for one time and three times, respectively. Obviously, it is not necessary to use medium with high sugar concentration in many fermentation. Thus, in spite that using dilute liquid enzyme for hydrolysis might result in lower sugar concentration obtained, the enzymatic hydrolysis cost could be saved by using lower amount of cellulase.

Analysis of sugar composition of enzymatic hydrolysate

Generally, the sugar composition might influence the later fermentation process. For example, many strains could not utilize xylose for growth and products accumulation (Rubin, 2008). Thus, after enzymatic hydrolysis, we further measure the sugar composition of the enzymatic hydrolysate by HPLC and the results were shown in Table 2.

As shown in Table 2, the enzymatic hydrolysate in this work mainly contained three kinds of sugar, namely, glucose, xylose, and cellobiose. The total sugar concentration of this hydrolysate was 93.7 g/L. As the substrate of enzymatic hydrolysis, composition of corncob was as follows: 56.7% cellulose, 31.5% hemicellulose, 7.1% lignin, and 4.7% others. As shown by the experimental results, the cellulose of corncob was completely degraded and most of hemicellulose was also hydrolyzed. After 72 h of hydrolysis, 65.9 g/L glucose and 20.0 g/L xylose were generated, and the hydrolysis yield could reach 95.7%.

Pretreated corncob was also hydrolyzed by other work using *Trichoderma reesei* ZU-02, and its hydrolysis yield was merely 67.5% (Chen et al., 2007). Although the hydrolysis rate could be improved to 83.9% by adding cellobiase from *Aspergillus niger* ZU-07 (Chen et al.,

2007), this would further increase the cost of enzyme and might not be suitable for the industrialization of bio-refinery. In contrast, in the present work, we merely used the liquid enzyme generated by *T. reesei* CH11, and this could save the cost in further application.

Conclusion

In this work, one promising method for cellulase generation and its enzymatic hydrolysis system was built in order to offer a suitable platform for bio-refinery. After enzymatic hydrolysis by *T. reesei* CH11, the hydrolysis yield could reach 95.7% and the total reducing sugars obtained were close to 100 g/L. All the fermentation substrates used in this work was low-cost one and also the liquid enzyme generated mixed with low-cost tap water was used for enzymatic hydrolysis. Overall, this process was green and easy for operation. Thus, it could be further applied in industrialization. Further work should focus on the pretreatment method to make the enzymatic hydrolysis more efficiency. Also, the modification of *T. reesei* is also necessary to have a higher hydrolysis yield.

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Optimization of bioplastic (poly-β-hydroxybutyrate) production by a promising *Azomonas macrocytogenes* bacterial isolate P173

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An extensive screening program was previously done to isolate a promising bacterial isolate *Azomonas macrocytogenes* isolate P173 capable of polyhydroxybutyrate (PHB) production. It produced 24% PHB per dry weight after 48 h. In this study, several experiments were designed to optimize the composition of the culture medium and environmental factors for maximizing PHB production by the respective isolate. Results show that 60% aeration, incubation temperature 37°C and an initial pH 7.5 were optimum for PHB production. A modified culture medium for PHB production was designed containing 0.7% glucose and 100 mg/L potassium nitrate as a carbon and nitrogen sources, respectively. Using this modified medium together with optimum environmental conditions, PHB production was increased from 24 to 42% per dry weight after 24 h of incubation rather than 48 h. Acriflavin-induced mutation resulted in one variant (173A2) which produced 47% PHB per dry weight after 24 h of incubation using the same modified culture medium except for glycerol 1.5% as carbon source.

Key words: *Azomonas macrocytogenes*, poly-β-hydroxybutyrate, optimization of microbial bioplastics bioplastic.

INTRODUCTION

Polyhydroxyalkanoates (biopolymers; PHA) are precious gifts of biotechnology to mankind. Since polyhydroxybutyrate (PHB) was discovered by Lemogine in *Bacillus megaterium* in 1926, there is extensive research towards industrial PHB production to substitute synthetic polymers (Anderson and Dawes, 1990). PHA resembles synthetic polymers in many chemical and physical properties, however being biodegradable and produced from renewable source makes it superior to its rival. They have a lot of applications, for example they are preferred candidates for developing controlled/sustained release drug delivery vehicles (Nair and Laurencin, 2007) and also can be used in biomedical implants and biofuels (Bonartsev et al., 2007; Zhang et al., 2009). However, PHB production is more expensive than synthetic polymer production so there is a need to explore its production from locally available and renewable carbon sources such as horticultural, agricultural waste, corn, cassava, etc. This would be of economic value considering the gains that would result from PHB application (Steinbüchel et al., 1998). Moreover optimization of other culture conditions such as the appropriate time to harvest polymer, aeration levels and incubation temperature is important to manage.

PHB has widespread occurrence in both Gram-positive and negative bacteria (Naranjo et al., 2013; Khanna and Srivastava, 2005; Anderson and Dawes, 1990). A lot of studies regarding PHB existence and optimization

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specifically in nitrogen fixing organisms were carried out. For example in free-living, N_2-fixing *Rhizobium* cells PHB synthesis has been described ranging from 35 to 50% per cell dry weight (Tombolini and Nuti, 1989). Moreover, Senior and his coworkers (1972) studied PHB production in *Azotobacter beijerinckii* which produced 50% PHB per dry weight with oxygen limitation conditions. Stockdale and his coworkers (1968) confirmed 22% PHB production per dry weight using 2% glucose as carbon source in *Azotobacter macrocytogenes*. Even mutant strain of *Azotobacter vinelandii* produced 65% PHB using glucose and ammonium acetate (Page and Knosp, 1989). An overall conclusion of PHB production is widespread in nitrogen fixing organisms.

In our previous study, a bacterial isolate from the soil was selected and its PHB production reached 24% per dry weight after 48 h of incubation. This isolate was fully identified using microscopical examination, culture characteristics, biochemical reactions and 16S ribosomal RNA gene sequencing (accession code KC685000) as *A. macrocytogenes* isolate P173 (Elsayed et al., 2013). *Azomonas* is nitrogen fixing Gram negative rod bacteria. To our knowledge, this is the first time to optimize PHB production in *Azomonas* species. Therefore, the resent work aims to study various environmental conditions and culture characteristics required to optimize PHB production by this isolate as well as strain improvement by mutation.

MATERIALS AND METHODS

Microorganisms

A. macrocytogenes isolate P173 (accession code KC685000) is a promising PHB producer obtained through an extensive screening from soil. The organism was maintained on nutrient agar slants at 4°C and renewed monthly.

Chemicals

Different chemicals used in the present study were of highest quality available and obtained mainly from Sigma-Aldrich (Munich, Germany), El-Nasr chemical Co. (Adwic, Cairo, Egypt) and other local suppliers. Ready-made culture media and media ingredients were obtained from Lab M (Topley house, England), Oxoid (USA) and Difco (Detroit, USA).

Culture Media

In the laboratory, formulated basal mineral salts medium (MSM; Berlanga et al., 2006), the modified MSM (M1, M2, M3 and M4) were used in this study and their compositions are listed in Table 1.

Fermentative production of PHB

The pre-culture was prepared by transferring a loopful from a culture grown onto nutrient agar into 5 ml LB broth (Miller, 1972), incubated at 37°C, at 160 rpm for 20 h. The production process was carried out in Erlenmeyer flasks (100 ml) containing 20 ml aliquots of MSM medium. The flasks were inoculated with the seed culture at 5% v/v (OD_{640nm} of 0.3) and incubated in a shaking incubator (200 rpm) at 37°C for 2 days. At different time intervals (time course experiments) or at the end the incubation period (other experiments), the fermentation broth was sampled to determine biomass and PHB concentration.

Analytical methods

Biomass determination

Cellular growth was expressed in terms of dry cell weight which was calculated from the equation of a calibration curve constructed between optical density (OD 640 nm) and dry cell weight of the tested isolate *A. macrocytogenes* isolate P173:

$$Y = 19.202X + 0.0782$$

Where, Y is OD 640 nm, X is dry cell weight (g %).

PHB concentration determination

For PHB extraction, an aliquot of 1 ml was withdrawn from MSM in Eppendorf tubes (1.5 ml) previously washed with alcohol followed by hot chloroform (to remove plasticizers) (Law and Slepeckey, 1961). The cells were harvested by centrifugation at 12,000 rpm for 5 min. The cell pellets were digested with a sodium hypochlorite solution (equivalent to active chlorine 4-6%; density at 20°C of 1.12) at 37°C for 1 h with stirring at 160 rpm. The insoluble materials containing PHB were collected by centrifugation at 12,000 rpm for 10 min. The pellets were washed with 1 ml aliquot each of water, ethanol and acetone, respectively. About 1 ml hot chloroform was added to extract PHB. After that 10 ml concentrated sulphuric acid (98%) were added to residue and the tubes were kept in a water bath at 100°C for 15 min. The solution was left to cooland the final solution was measured with a spectrophotometer at 235 nm.

Studying the different environmental and physiological conditions

For each condition, the biomass, PHB concentration and itsproduction percent per dry weight for the test isolate were measured as previously mentioned.

The effect of aeration

This was done using Erlenmeyer flasks (250 ml) with the following varying volumes of MSM: 25, 50 and 100 ml corresponding to 90, 80 and 60% aeration (Park et al., 1997).

The effect of inoculum size

Erlenmeyer flasks (100 ml) each containing 20 ml MSM were prepared and inoculated with pre-culture with different inoculum sizes (0.5, 2, 5 and 10 % v/v).

The effect of initial pH

Erlenmeyer flasks (100 ml) each containing 20 ml MSM medium were prepared at different pH values of 4, 5, 7, 8 and 9.

Table 1. Chemical composition of MSM and modified media.

Name of ingredient	Basal medium (MSM)	Modified media			
		M1	M2	M3	M4
Carbon source (amount/liter)	Glucose (7 g)	Glucose (7 g)	Glycerol (15 ml)	Glucose (7 g)	Glycerol (15 ml)
Nitrogen source (amount/liter)	Ammonium chloride (100 mg)	Potassium nitrate (100 mg)	Potassium nitrate (100 mg)	Potassium nitrate (100 mg)	Potassium nitrate (100 mg)
Minerals (amount/liter)	$MgSO_4 \cdot 7H_2O$(0.2 g); $CaCl_2$ (0.01 g); Ferrous ammonium sulphate (0.06g); Trace elements solution* (1 ml)	No tested minerals		Same minerals as in MSM	
Common ingredients (amount/liter)	$Na_2HPO_4 \cdot 12H_2O$ (10.2 g); KH_2PO_4 (1.5 g); NaCl (10 g)	The same as in MSM			

*Trace elements solution contains (amount/liter) ($CoCl_2.6H_2O$ (0.2 g), H_3B0_3 0.3 g, $ZnSO_4.\ 7H_2O$ 0.1 g, $MnCl_2\ .4H_2O$ 30 mg, $NiCl_2$ 10 mg, $CuSO_4.\ 5H_2O$ 10 mg) (Berlanga et al., 2006); MSM and modified media were sterilized by autoclaving. Glucose and trace elements solution were filter sterilized and were aseptically added to the autoclaved media with the indicated concentration.

The effect of incubation temperature

Erlenmeyer flasks (100 ml) each containing 20 ml MSM were prepared, inoculated and incubated at different temperatures (28, 37 and 40°C).

The effect of different media components

Effect of replacement of glucose in MSM with other carbon sources: A set of flasks with MSM media were prepared with same ingredients except for replacement of glucose with other carbon sources (0.7%). The used carbon sources were classified as follows: monosaccharaides sugars (fructose, galactose and, arabinose), disaccharides sugars (maltose, lactose, sucrose), sugar alcohol (mannitol, glycerol), polysaccharides as starch, oils (paraffin, corn oil), unrefined carbon sources as Malt extract and others as potassium acetate. All carbon sources were prepared as stock solution (10%) and sterilized by autoclaving except for fructose and arabinose which were sterilized by membrane filtration.

At the end of incubation, samples were removed to measure biomass and PHB concentration. Except in the case of using oils as carbon source, biomass was measured as optical density and expressed in terms of dry weight as previously described. In case of oils, formation of emulsion between oil and medium confers turbidity to medium which affects optical density. In this case, biomass was measured directly by drying 5 ml culture in pre-weighed centrifuge tubes at 37°C for 24 h.

Effect of variable concentration of some selected carbon sources in MSM: The carbon sources showing promising PHB percentage per dry weight without abrupt decrease in biomass were tested in different concentration to determine the most suitable concentration to be used in further experiments. The concentrations used were 0.4, 0.7, 1.5, 2 and 4% w/v.

Effect of replacement of ammonium chloride in MSM with other nitrogen sources: Erlenmeyer flasks (100 ml) each containing 20 ml MSM were prepared with the same ingredients except for replacement of ammonium chloride with other nitrogen sources (100 mg/L). The nitrogen sources used were organic ones as yeast extract, peptone, tryptone, beef extract, urea and amino acids and inorganic ones as ammonium nitrate and potassium nitrate.

All nitrogen sources were sterilized by autoclaving except for urea which was prepared as stock solution and sterilized by membrane filtration.

Effect of variable concentration of some selected nitrogen sources

The nitrogen sources showing promising results were tested at different concentrations 50, 100, 200, 500 and 1000 mg/l to determine the best suitable concentration to be used in further experiments.

Effect of different multivalent minerals

Initially formulated MSM contain ferrous ammonium sulphate, calcium chloride, magnesium chloride and trace elements. To study the effect of these minerals both on biomass and PHB productivity, five flasks each containing 20 ml MSM were prepared as follows: One is MSM devoid of all minerals; second contained only ferrous ammonium sulphate; third contained only calcium chloride; fourth contained only magnesium chloride and fifth contained only trace elements solutions.

The used mineral concentrations were the same as in MSM medium.

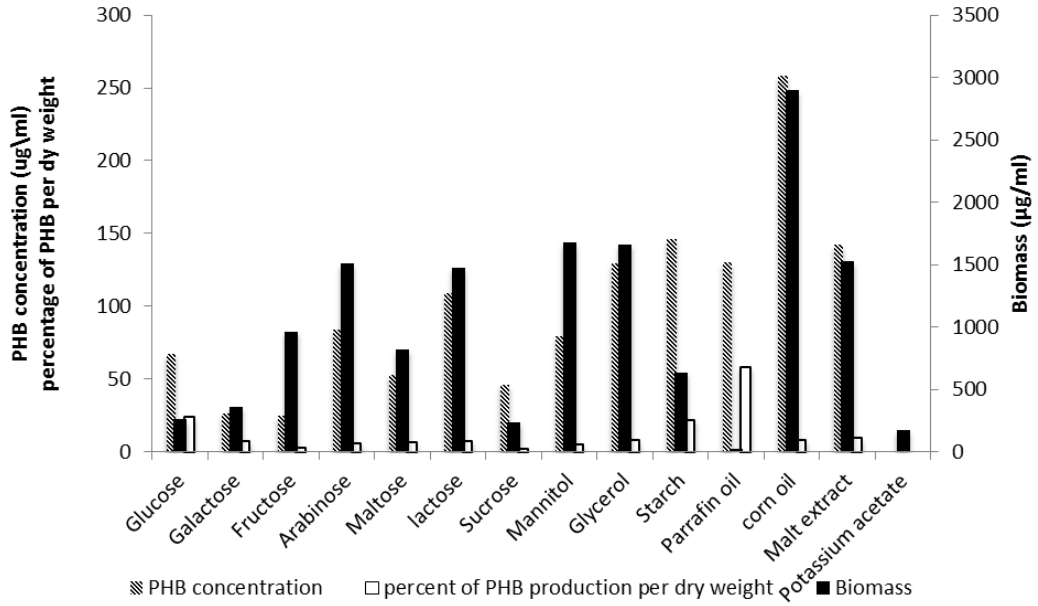

Figure 1. Effect of different carbon sources on PHB concentration, biomass and PHB percentage per dry weight in *A. macrocytogenes* isolate P173.

PHB production under combined selected pre-tested conditions

From the previously studied factors/conditions that proved to be optimium for PHB production by our isolate, four modified media were created. In most cases, the selection criteria for the chosen factors/conditions depended on high PHB percentage per dry weight and reasonable amount of biomass formation not less than 50% of the maximum biomass attained by different factors within the same tested category. The modified media were M1, M2, M3 and M4 (Table 1). Time course of PHB production was done after 24, 48 and 72 h.

Induction of mutation using UV rays

This was done by Direct-Plate Irradiation (Lin and Wang, 2001). A loopful of isolate from a nutrient agar slant was inoculated in LB broth for overnight at 37°C and 200 rpm. A series of dilutions of the obtained culture were used to prepare two sets of nutrient agar plates. One set for UV irradiation and the other for estimating viable count in the bacterial culture. All UV irradiations were done in a custom-built UV chamber with a glass front (germicidal lamp 15 W UV lamp of 254 nm). The majority of the experiments were done in the dark to avoid photo reactivation. All the plates were grown in the 37°C incubator for 24 h before scoring the number of colonies. This treatment resulted in 99.9% kill as determined by viable count of the survivors. The resultant colonies were collected for PHB production assessment.

Induction of mutation using chemical mutagens

This was done according to Lopes et al. (2010) with minor modifications. Stock solutions of acriflavin and proflavin (each of 50 mg/ml) were prepared in phosphate buffer (50 mM, pH7.5) and sterilized by membrane filtration. The isolates were grown by inoculating a loopful of nutrient agar slant in LB broth (10 ml final volume) for overnight at 37°C and 200 rpm. Aliquots of 0.5 ml from stock solutions of acriflavin and proflavin were separately added to equal aliquots of cell suspension of isolates. The contact time of reaction of chemical mutagen and cell suspension was 30 min. Then, the reaction mixtures were centrifuged at 12000 rpm for 10 min and the cells were washed with normal saline. The mutagen treated cells were re-suspended in 200 µl sterile saline and surface inoculated on nutrient agar plates.The plates were incubated at 37°C for 24 h. The grown colonies were collected to measure PHB production.

Testing the PHB productivity of the collected variants

Screening for PHB was done for the collected variants of our isolate as previously described. Comparison of the PHB production of the collected variants to its wild type organism was carried out.

RESULTS

Effect of environmental factors on PHB production

As shown in Table 2, maximum PHB percentage per dry weight was achieved at 80% aeration, 5% inoculum size, pH of 7.5 and 37°C incubation temperature.

Effect of different media components on PHB production

Effect of carbon sources

As shown in Figure 1, the highest PHB percentage per dry weight was attained using paraffin oil (42%). Paraffin

Table 2. Effect of aeration, inoculum size, initial pH and incubation temperature on PHB concentration, biomass and PHB percentage per dry weight in *A. macrocytogenes* isolate P173.

Factor	PHB (µg/ml)	Biomass (µg/ml)	PHB percentage per dry weight(%)
60% Aeration	24	383	6
80% aeration	184	1400	13
90% aeration	155	366	42
0.5% inoculum size	55	580	9.5
2% inoculum size	123.5	615	15.7
5% inoculum size	169	770	24
10% inoculum size	148	840	17.6
28°C	74	820	9
37°C	169	667	25
40°C	92	870	10.5
pH 4	0	257	0
pH 5	71	766	9.2
pH 7.5	169	667	25.3
pH 8	121	603	21.7
pH 9	127	988	20

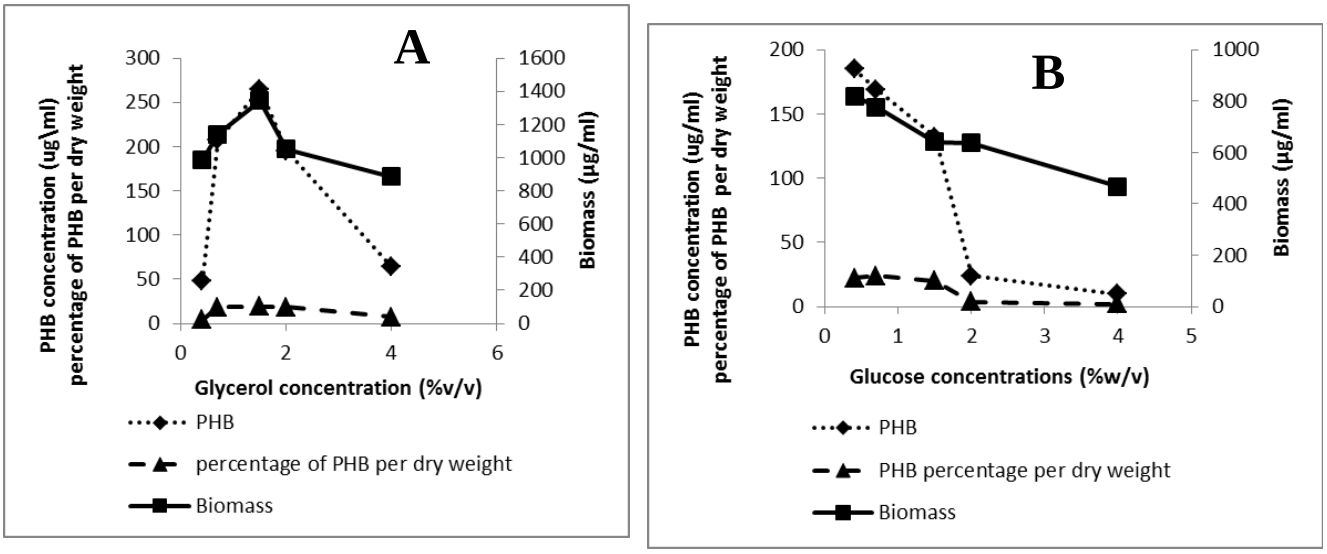

Figure 2. Effect of different concentrations of glycerol (A), glucose (B) on PHB concentration, biomass and PHB percentage per dry weight in *A. macrocytogenes* isolate P173.

oil decreased biomass to a great extent while producing a moderate amount of PHB thus increasing PHB percentage per dry weight. Glycerol, glucose, malt extract and corn oil increased amount of PHB produced much more than paraffin oil but they did not decrease biomass so they gave lower PHB percentage per dry weight. Therefore, different concentration of glucose and glycerol were tested as they produced the largest PHB percentage per dry weight without decreasing biomass to less than 50% attained with other carbon sources. Figure 2A reveals that 0.7% w/v glucose led to highest PHB percentage per dry weight (24%) without affecting biomass, also 1.5% v/v glycerol gave highest amount of PHB but the biomass was increased to a large extent leading to obvious decrease in PHB percentage per dry weight (19%) (Figure 2B).

Effect of nitrogen sources

Figure 3 shows that maximum PHB production percentage per dry weight was achieved using ammonium

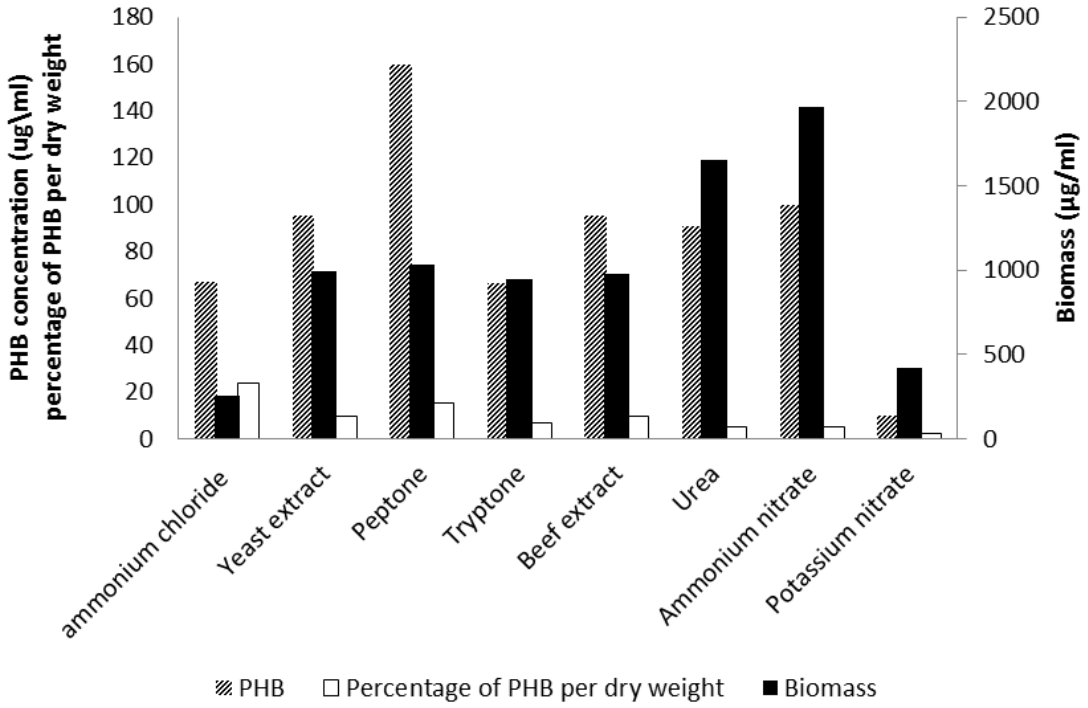

Figure 3. Effect of different nitrogen sources on PHB concentration, biomass and PHB percentage per dry weight in *A. macrocytogenes* isolate P173.

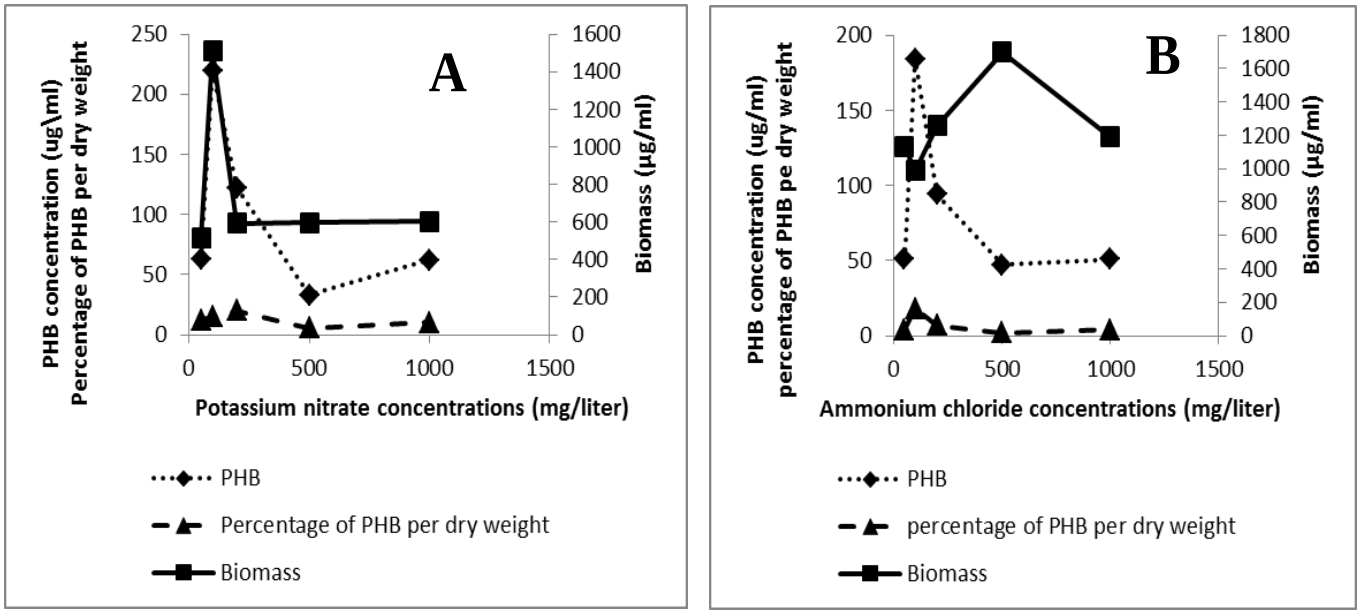

Figure 4. Effect of different concentrations of potassium nitrate (A), ammonium chloride (B) on PHB concentration, biomass and PHB percentage per dry weight in *A. macrocytogenes* isolate P173.

chloride followed by potassium nitrate. Therefore, different concentrations of both potassium nitrate and ammonium chloride were tested (Figure 4). Results revealed that 100 mg/L potassium nitrate gave the highest PHB percentage per dry weight without decreasing biomass.

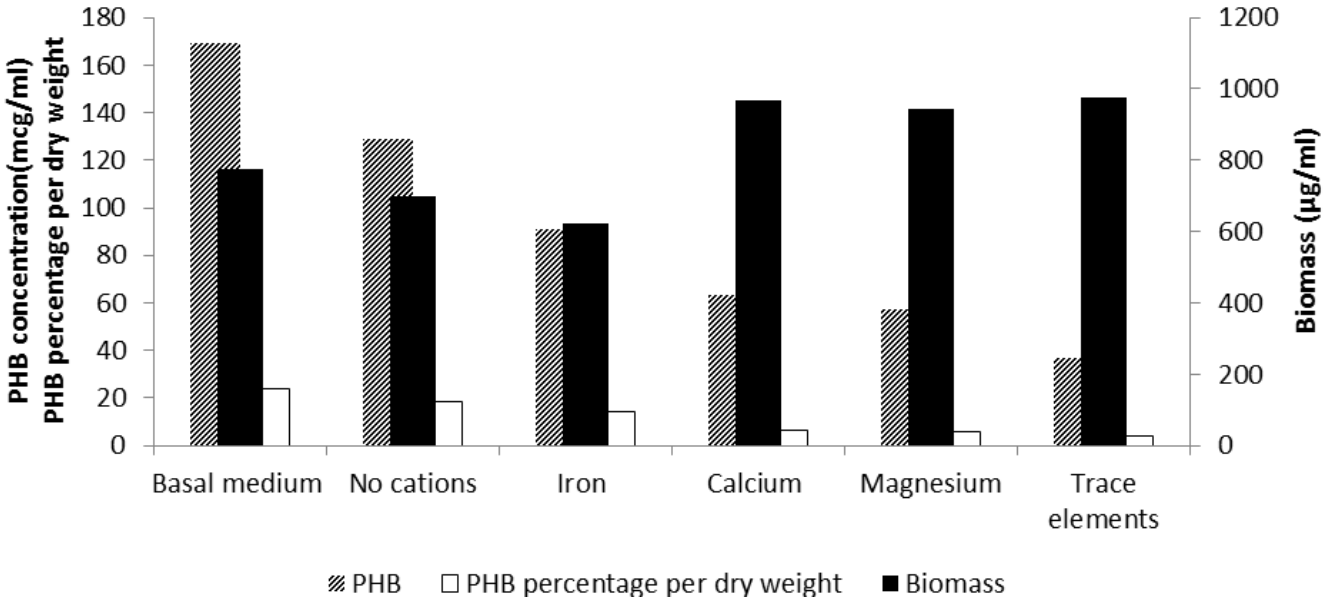

Figure 5. Effect of minerals on PHB concentration, biomass and PHB percentage per dry weight in *A.* macrocytogenes isolate P173.

Table 3. Comparison of maximum productivity and PHB production percentage per dry weight of *A. macrocytogenes* isolate P173 in basal and newly formulated media.

Name of medium	Maximum PHB production (µg/ml) (time in hours)	Maximum PHB percentage per dry weight (%) (time in hours)
Basal medium (MSM)	172 (48)	23 (48)
173M1	230 (24)	42 (24)
173M2	143 (24)	10 (24)
173M3	177 (48)	13.8 (48)
173M4	137 (48)	15.8 (48)

Effect of minerals

Highest PHB production percentage per dry weight was attained using MSM containing all minerals rather than their absence or their separate usage in medium (Figure 5).

PHB production under combined selected pre-tested conditions

As depicted in Table 3, PHB production in medium 1 (M1) reached 42% per dry weight after only 24 h of incubation in comparison with MSM medium. This means two fold increase in PHB percentage per dry weight. The other modified media M2, M3 and M4 show no significant effect on PHB production. The time course of PHB production using the best modified medium 173M1 is shown in Figure 6.

Effect of mutation

Acriflavin-induced mutation resulted in variant 173A2 which produced more PHB than wild type (P173). However, UV induced mutation as well as proflavin-induced mutation resulted in decreasing PHB production in the collected variants (Figure 7). Therefore, variant P173A2 was chosen for further experiments.

Testing PHB productivity by *Azomonas macrocytogenes* variant P173A2 in the four modified media

The variant P173A2 accumulated PHB up to 47% per dry weight after 24 h of incubation in medium 2 (M2). This differs from wild type which produced only 10% PHB per dry weight using this medium. The other modified media showed no significant effect on PHB production by this

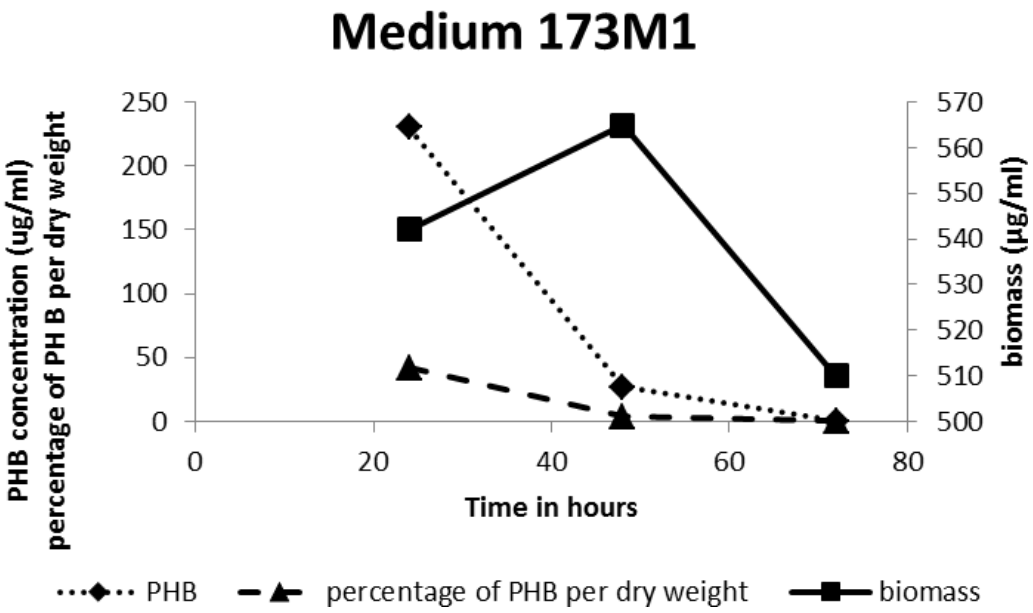

Figure 6. Time course of PHB production, biomass and PHB percentage per dry weight using the best modified medium (173M1) for *A. macrocytogenes* isolate P173.

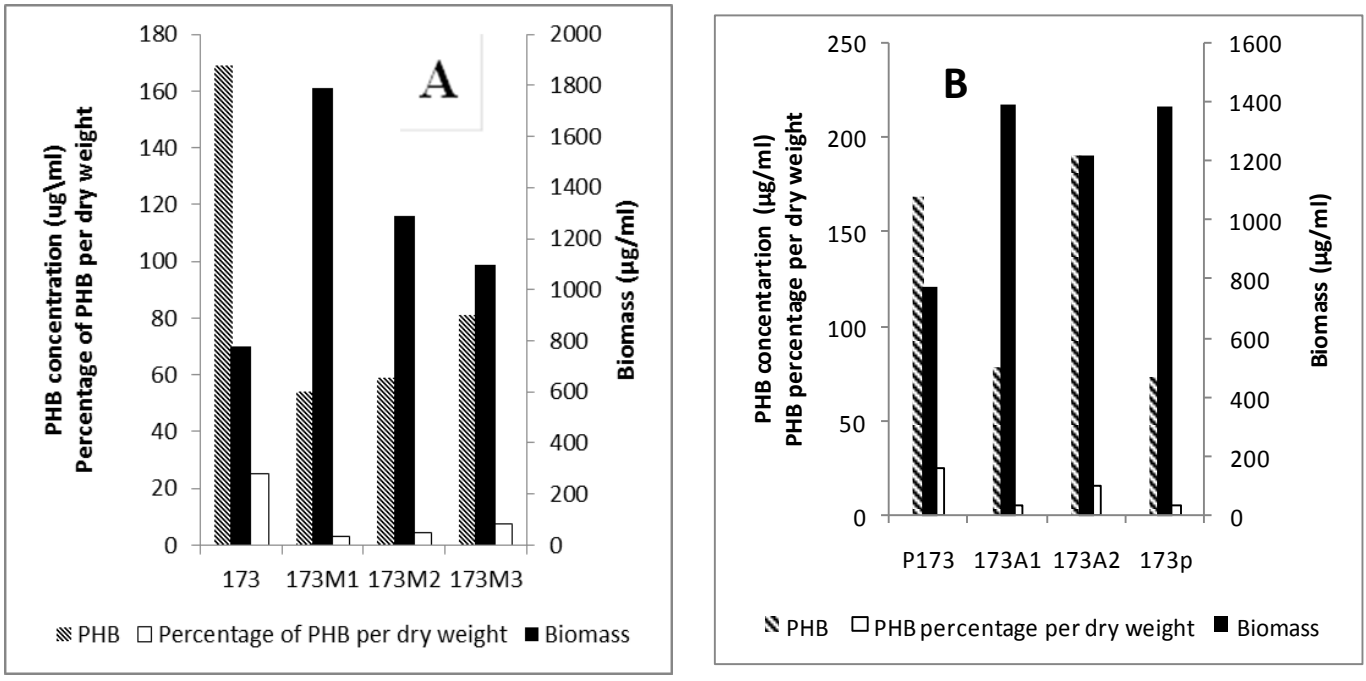

Figure 7. Results of screening of PHB production of *A. macrocytogenes* isolate P173 variants after exposure to UV (A), chemical mutagens (B).

variant. The comparison between four modified media in terms of maximum PHB productivity is shown in Table 4. Time course of PHB production using best modified medium for the variant 173M2 is shown in Figure 8.

DISCUSSION

A. macrocytogenes is ellipsoidal to rod-shaped Gram negative cell that is more than 2 mm in diameter, usually

Table 4. Comparison of maximum productivity and PHB production percentage per dry weight of *A. macrocytogenes* variant 173A2 in newly formulated media.

Name of medium	Maximum PHB production (µg/ml) (Time in hours)	Maximum PHB percentage per dry weight (%) (time in hours)
173M1	124 (24)	23.9 (24)
173M2	212 (24)	47 (24)
173M3	156 (72)	13.6 (72)
173M4	187.5 (24)	15 (72)

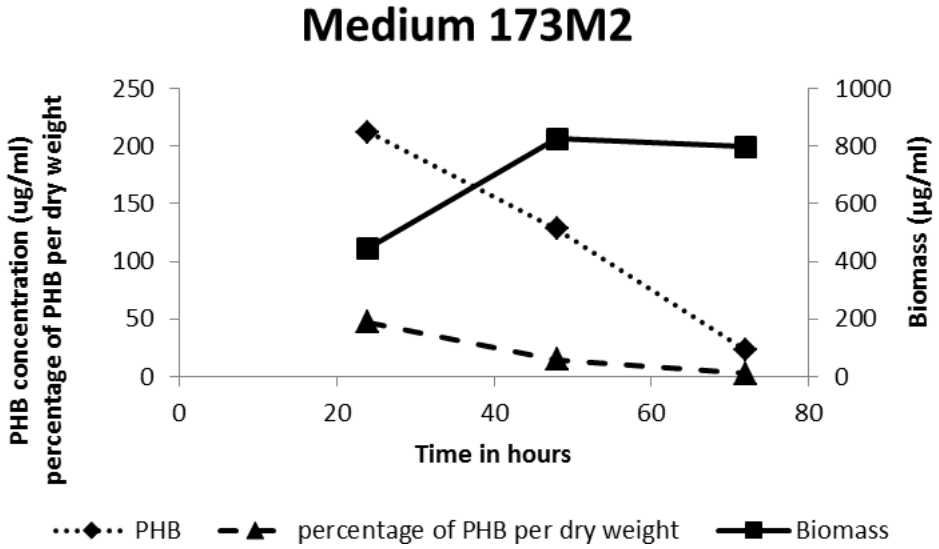

Figure 8. Time course of PHB production, biomass and PHB percentage per dry weight using the best modified medium for *A. macrocytogenes* variant P173A2.

2.5-3.5 µm in length, motile by peritrichous, lophotrichous or polar flagella and is capable of fixing nitrogen under aerobic conditions (Young and Park, 2007). In this study, an optimum medium for PHB production for this species was created using a series of experiments. We found that 80% aeration, pH of 7.5, incubation temperature 37°C were the best environmental conditions for PHB production in this bacteria. Also, the best modified culture medium should consist of 0.7% glucose, 100 mg/L potassium nitrate and absence of tested minerals. These conditions encouraged bacteria to produce 42% PHB per dry cell weight after only 24 h of incubation. These minimum requirements in culture medium and short time of incubation make PHB production in *A. macrocytogenes* a cost-effective process. Moreover, after exposing this bacterium to acriflavin as a chemical mutagen, the collected variant (P173A2) showed improvement in PHB production. This variant (P173A2) produced 47% PHB per dry cell weight after 24 h of incubation using the same culture medium as for the wild type except for glycerol as carbon source.

Among the investigated parameters was aeration percentage, it was found that maximum biomass as well as PHB production occurred at 80% aeration, however, both decreased at higher aeration percentage. Barron (1955) proposed that oxygen may have harmful effect on biochemical materials due to nonspecific oxidation of enzymes (Barron, 1955).

After studying the effect of pH on PHB production, we found that maximum PHB percentage per dry weight occurred at pH of 7.5. This agreed with many previous studies (Grothea et al., 1999; Tamdogan and Sidal, 2011). At extremes of pH, PHB production decreased in spite of increasing biomass (Tavernier et al., 1997). We speculated that *Azomonas* directed its energy for growth rather than PHB production.

Maximum PHB production was achieved at temperature 37°C. A lot of studies confirmed maximum PHB production at range of 33-37°C (Grothea et al., 1999; Tabandeh and Vasheghani, 2003). It was pointed out that PHB production decreased at temperature extremes due to low enzyme activity at such temperatures (Tamdogan and Sidal, 2011).

The inoculum size of seed culture was investigated to

stabilize initial microbial load. 5% v/v was the best initial inoculum size. Furthermore, different classes of carbon source were studied to point out the best carbon to be used in optimized medium. *Azomonas* produced maximum PHB from monosaccharides followed by sugar alcohols followed by oils. Moreover, *Azomonas* showed a low efficacy in utilizing disaccharides sugar and polysaccharides. Maximum PHB was achieved using 0.7% glucose without affecting biomass. A lot of studies are comparable to our results; glucose is an easily assimilated carbon source and encouraged bacteria to produce PHB (Ramadas et al., 2009; Hori et al., 2001; Borah et al., 2002).

It is well known that any bacteria capable of producing PHB needs excess carbon source in addition to a limited other source such as nitrogen or phosphate (Naranjo et al., 2013; Santhanam and Sasidharan, 2010). Here, we used nitrogen source as the limiting one. Our bacteria synthesized PHB in large amounts using inorganic nitrogen sources rather than organic ones. The best nitrogen source used was potassium nitrate. This agreed with some studies especially using glucose as carbon source (Pal et al., 2008; Rohini et al., 2006).

It is fascinating that our newly modified medium helped the bacteria to produce double its initial PHB production from MSM medium from 24 to 42% per dry cell weight. Also, maximum PHB as well as biomass occurred after 24 h of incubation. Afterwards, PHB decreased dramatically due to its consumption by the bacteria. Naheed et al. (2011) stated that 66% PHB per dry weight was achieved by *Enterobacter* after 24 h of incubation. Also, Rohini et al. (2006) reported 19.7% PHB per dry weight using *Bacillus thuringiensis*. Kim (2000) mentioned that *Azotobacter* produced 46% PHB per dry weight using starch.

In order to complete our study, strain improvement by mutation was tried both physically and chemically. Physical mutation was done by UV where UV induces a complex spectrum of mutations (Miller, 1985). We exposed our isolate to UV rays but unfortunately no improvement in PHB production has occurred, although a lot of studies evaluated UV as a mutagen for PHB production and successfully managed to reach its target (Pal et al., 2008; Sreeju et al., 2011). On the other hand, after exposure of our isolate to chemical mutagen especially acriflavin, one of the collected variant improved PHB production. Chemical mutations not only by acriflavin, but also nitrosoguanidine or ethylmethylsulphonate are known for improving PHB production (Lakhawat et al., 2012; Pal et al., 2008; Sreeju et al., 2011). We tested the four optimized media with this variant, medium 2 (M2) proved to be effective with it which composed of glycerol instead of glucose. It is known that glycerol is cheaper than glucose (Naranjo et al., 2013). This strengthens our target to decrease cost of PHB production. Moreover, it produced 47% PHB per dry weight after 24 h of incubation. Therefore, the prospective work will be directed to large scale production of PHB using *A. macrocytogenes* isolate P173 and molecular characterization of the genes involved in the biosynthesis of PHB.

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Full Length Research Paper

Mycoflora associated with processed and stored cassava chips in rural areas of southern Cameroon

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A total of 132 home-stored samples of cassava chips were collected in 45 villages situated in three distinct geographical locations of Southern Cameroon for assessment of their mycoflora. Cassava chips were split into small pieces (10 x 10 x 5 mm) and these were plated onto Petri dishes containing water agar at 25°C after a 7 day incubation period to yield of mycoflora. *Aspergillus*, *Fusarium* and *Penicillium* spp. were the main groups of fungi isolated. 14 *Aspergillus*, nine *Fusarium* and 14 *Penicillium* spp. were identified from a total of 3204 isolates obtained. *Aspergillus* spp. ranked first in prevalence at 57%. *Penicillium* spp. represented only 14% of all isolates. Six stored product-insects in five families and two orders were found infesting some samples. These did not appear to have any significant (*P*< 0.05) relationship with the level of recovery of the fungal taxa detected. Only the moisture content and the location of sample collection had statistically significant (*P*< 0.05) impact on fungal infestation of samples. Diversity indices computed using Simpson's index for all fungal genera and for each location showed that these varied greatly. Across locations, Mbalmayo and Yaoundé were sources of greater species diversity. Among the three fungal groups, *Aspergillus* species yielded the most diverse fungal population. The results of the present study could provide a basis for identifying and selecting specific fungi, especially toxigenic species for which intensive efforts should be directed to assess potential mycotoxin problems occurring alone or in combination on stored cassava products.

Key words: *Aspergillus*, *Fusarium*, *Penicillium*, diversity indices, mycotoxin.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz, *Euphorbiaceae*) is one of the most important food staples of Cameroon where it is grown in association with several other food crops such as maize (*Zea mays* L. *Poaceae*), yams (*Dioscorea* spp. L. *Dioscoreaceae*), and sweet potatoes (*Ipomea batata*s L. *Convolvulaceae*). In almost all the cassava growing communities of Africa, the roots (the most widely edible organs of cassava) are consumed mainly in processed forms.

Cassava roots are low in protein, but very high in carbohydrates. Its leaves have a relatively higher protein, and calcium content (Cock, 1985). Further, rootsand leaves of some cultivars tend to be high in cyanogenic compounds (Essers, 1995). In this respect, research has established that cassava processing could successfully reduce or eliminate the cyanogenic compounds found in both the roots and leaves (Bokanga, 1996; Hahn, 1993). However, some authors have reported the association of several micro-organisms with cassava processing, among them are toxigenic taxa. Westby and Twiddy (1991) documented the spectrum of bacteria contaminating gari, a fermented cassava product widely consumed in Western and Central Africa. Among them were toxin-producing species, especially spore-forming

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specimen such as *bacillus* spp. Similarly, Essers (1995) isolated 19 fungal species from on-farm fermented cassava products from Uganda and Mozambique, with toxigenic genera such as *Penicilium* and *Aspergillus* being the most representative.

In Cameroon, surveys recently conducted in rural areas indicated that cassava chips, a cassava product obtained after peeling, fermentation, and drying of fresh roots,were the raw material for the bulk of cassava-based foods in the country (Essono, 2008) as it is the case for the majority of African countries where the crop forms the staple (Ugwu and Ay, 1992). In the frame of these surveys, observations were made that chips could be stored for periods exceeding 180 days prior to consumption. In addition, several processing and storage practices were observed, which could impact the microflora profile of the products.

Previously, a study on the microbial composition of derived cassava products sold in market places of former Zaïre (present day Democratic Republic of Congo) reported the occurrence of toxigenic *Penicillium* and *Aspergillus* spp. from an unspecifiednumber of samples analyzed (Liya et al., 1985). These findings are consistent with preliminary results obtained later by Msikita (1995) and Msikita et al. (1998, 2001), while investigating the species composition of the mycoflora of stored cassava chips sampled in Benin and Nigeria.

Although, the three studies mentioned above provided some useful background information with respect to the species composition of the mycoflora of the chips, no mention was made of the processing and storage practices under which the samples examined were collected. In Cameroon, no comprehensive survey has been carried out on the fungal flora of derived cassava products and especially cassava chips.

In this study, three locations covering 45 villages were surveyed to obtain quantitative and qualitative data on the main fungi associated with cassava chips in storage. Due to their relative toxigenicity, emphasis was placed on *Penicillium, Aspergillus* and *Fusarium*. In addition, attempts were made to relate the effects of some intrinsic parameters, such as moisture content and levels of insect infestation on the mycoflora composition associated with the samples collected across the locations investigated. Diversity associated with these three fungal genera was assessed using frequencies of occurrence computed on the basis of total isolation figures associated with each of them in the three locations of sample collection.

MATERIALS AND METHODS

Survey areas and samples collection

Surveys were undertaken at household level from December 2010 to June 2011 in 45 villages in Yaoundé, Mbalmayo and Ebolowa: three locations referred to as blocks and situated in southern Cameroon, where cassava is intensively cultivated and processed, and were previously identified as ecologically distinct (Figure 1) (Anonymous, 1996).

The Yaoundé (Yao) block lies within latitude 3°45' to 4°26'N and longitudes 11°14' to 11°35'E and covers an area of 5200 km^2. This block has an annual mean temperature of 22.2°C and a relative humidity of 77.7%. The Mbalmayo(Mba) block stretches from latitudes 3°16' to 3°37'N and longitudes 11°6' to 11°47'E, covering 5120 km^2. In this block, the average relative humidity is 79% and the annual mean temperature is 23°C. The Ebolowa(Ebo) block covers a surface area of 5150 km^2 and stretches from the southern boundary of Cameroon to Gabon and Equatorial Guinea, within latitudes 2°20' to 3°5'N and longitudes 11°00 to 11°24'E. The block has an average relative humidity of 83.4% and an annual mean temperature of 24.4°C. In all blocks, the annual rainfall is distributed in a bimodal pattern with the greatest accumulation rate in September-October and April, May, averaging 1654 mm in Yaoundé, 1624 mm in Mbalmayo and 1876 mm in Ebolowa.

These surveys were aimed at collecting production practices information from farmers producing cassava chips and to assess the fungal flora composition of such chips. Within each block, 15 villages were selected on the criteria that socio-economic and biophysical characteristics studies have been carried out therein by scientists of the International Institute of Tropical Agriculture (IITA). Three households were randomly selected along a transectcutting across each of the selected villages. From each household, home-storedsamples of cassava chips of any types (balls or pellets) each weighing about 4 kg were hand-collected.

Collection of cassava chips was done from the top to the bottom of the sample package so as to obtain a composite sample. These were sealed in polyethylene bags, labelled, placed in a cooler, and brought to the laboratory for mycological analyses. A total of 42 samples were collected in the Yaoundé block, and 45 samples for each of Mbalmayo and Ebolowa. An aggregate of 132 composite samples were thus obtained. The type of chips produced in each location, the methods implemented for their production, and the storage facilities used for their preservation were recorded and are presented in Table 1.

Preparation of cassava chips samples

In the laboratory, samples of cassava chips were divided into four 1 kg batches. The first batch was kept aside as a backup; the second was directly used to determine the water content. The third was used to estimate the insect population per unit of weight of cassava chips by sieving. The fourth for mycological analyses was preserved in sterile plastic bags at 4°C in a cold chamber. The maximum preservation period was 6 weeks.

Moisture content determination and assessment of insect infestation

At collection period, three 100 g samples were weighed out from each second batch of cassava chips using an analytical balance (Model: Sar CP 225D). Samples were subsequently dried in an oven (Model: Gallenkamp Plus II) at 60°C for 72 h. Moisture contents were hence determined on a weight loss basis.

Insect infestation was assessed by sieving three 100 g samples of cassava chips showing visible holes from the fourth batch with a 2.00 mm mesh sieve. Insects (live and dead adults) were collected in a second sieve of 1.00 mm mesh and numbers were recorded. The insects recovered were shipped to the entomology museum at IITA Cotonou (Plant Health Management Division) for identification.

Isolation and identification of fungal flora from cassava chips

To identify fungal flora, cassava chips arbitrary selected from batch

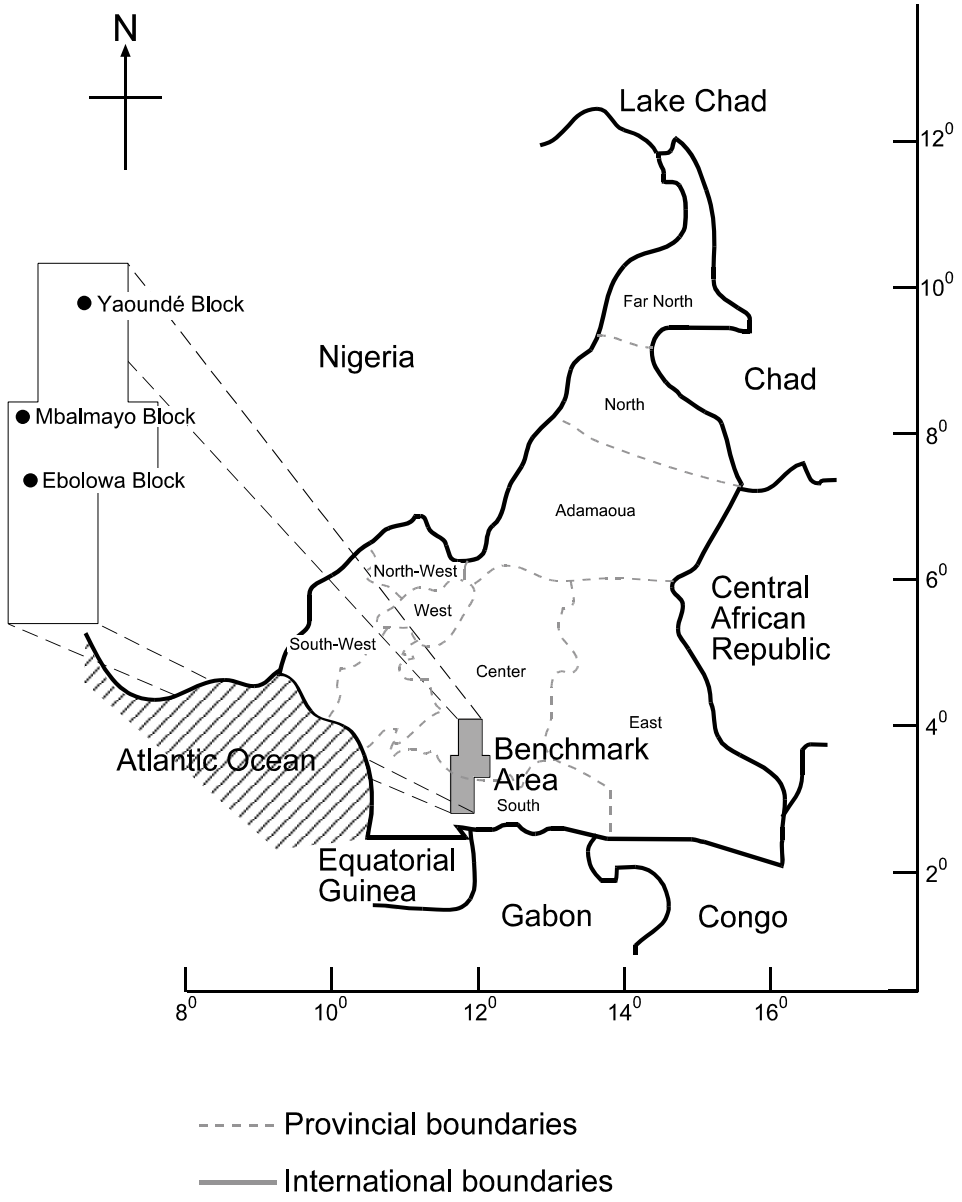

Figure 1. Study site indicating the 3 locations of sample collection.

4 of each sample package were split with a flamed knife into small pieces (10 x 10 x 5 mm) and these were placed in the laminar flow cabinet. Inside the laminar flow, five pieces were removed with sterile forceps and plated in triplicates onto 9 cm diameter Petri dishes (at the center and across two perpendicular diameters of the Petri dishes) containing 20 ml of water agar (BDH Laboratory Supplies) adjusted to pH 4.5 with 0.1 M sulfuric acid to suppress bacterial growth. Preliminary studies (data not shown) carried out on a few samples in the frame of this study using surface sterilization with NaClO as suggested by Msikita (1995), hardly allowed the recovery of fungi from cassava chips' samples even when such samples were observed overgrown with fungi. We attributed this to the lack of a protective shell in cassava chips as is the case for food products such as maize, groundnuts or rice. The presence of a protective barrier is useful in limiting or preventing the sterilizing substance used at appropriated concentration from diffusing into the inner parts of the analyzed product and killing the internal fungi. In addition, heat sterilization further used did not show any significant differences in the recovery of the fungal species when compared with the non-sterilization method finally adopted in this study. Accordingly, the pieces of cassava chips were not subjected to any prior disinfection. The plates were incubated for seven days at 25°C. Isolated fungi were further sub cultured and purified using single spore cultures onto one-quarter (1/4) strength PDA (Potato Dextrose Agar).

Synoptic keys used in mycology (Raper and Thom, 1949; Raper and Fennel, 1965; Barnet and Hunter, 1972; Nelson et al., 1983; Singh et al., 1991) were employed to identify the fungal isolates recovered from samples. The fungi detected were grouped into similar categories such as *Aspergillus* spp*., Penicillium* spp*., Fusarium* spp*.,* and other miscellaneous fungi. Species initially suspected as belonging to *Penicillium* spp*., Paecilomyces* spp. and

Table 1. Main characteristics of samples of cassava chips collected in three locations.

Location	Chip type	Number of samples[a]	Processing methods[b]	Storage facilities[c]	Storage duration[d]
Yaoundé	Pellets	07	1	Opened containers	< 14 days
Yaoundé	Pellets	05	1	Opened containers	[14-45 days]
Yaoundé	Pellets	01	1	Opened containers	> 60 days
Yaoundé	Pellets	01	2	Closed containers	< 14 days
Yaoundé	Pellets	01	1	Closed containers	< 14 days
Yaoundé	Pellets	02	1	Closed containers	[14-45 days]
Yaoundé	Pellets	03	1	Plastic-jute bags	< 7 days
Yaoundé	Pellets	01	1	Plastic-jute bags	[14-45 days]
Yaoundé	Pellets	10	3	Jute bags	< 14 days
Yaoundé	Pellets	02	1	Jute bags	< 14 days
Yaoundé	Pellets	03	1	Jute bags	[14-45 days]
Yaoundé	Pellets	04	1	Jute bags	> 60 days
Yaoundé	Balls	01	1	Over fireplace	< 14 days
Yaoundé	Balls	01	2	Over fireplace	[14-45 days]
Mbalmayo	Pellets	10	2	Opened containers	< 14 days
Mbalmayo	Pellets	03	3	Opened containers	< 14 days
Mbalmayo	Pellets	07	1	Opened containers	[14-45 days]
Mbalmayo	Pellets	05	3	Opened containers	[14-45 days]
Mbalmayo	Pellets	03	1	Opened containers	> 60 days
Mbalmayo	Pellets	03	1	Closed containers	< 14 days
Mbalmayo	Pellets	02	1	Closed containers	[14-45 days]
Mbalmayo	Pellets	01	3	Closed containers	[14-45 days]
Mbalmayo	Pellets	05	1	Plastic-jute bags	> 60 days
Mbalmayo	Pellets	02	3	Plastic-jute bags	[14-45 days]
Mbalmayo	Pellets	01	1	Jute bags	< 14 days
Mbalmayo	Pellets	01	1	Jute bags	[14-45 days]
Mbalmayo	Pellets	01	1	Jute bags	> 60 days
Mbalmayo	Balls	01	1	Over fireplace	[14-45 days]
Ebolowa	Pellets	07	2	Opened containers	< 14 days
Ebolowa	Pellets	04	3	Opened containers	[14-45 days]
Ebolowa	Pellets	01	1	Closed containers	[14-45 days]
Ebolowa	Pellets	03	1	Jute bags	< 14 days
Ebolowa	Pellets	01	3	Jute bags	< 14 days
Ebolowa	Pellets	04	1	Jute bags	[14-45 days]
Ebolowa	Balls	05	2	Over fireplace	< 14 days
Ebolowa	Balls	11	2	Over fireplace	[14-45 days]
Ebolowa	Balls	05	2	Baskets	< 14 days
Ebolowa	Balls	04	2	Baskets	[14-45 days]

[a]Total number of samples: Yaoundé = 42; Mbalmayo = 45; Ebolowa = 45. [b]Processing methods: 1: Steeping washed and peeled cassava roots in water for “to 4 days before drying under sunlight for 2 days. 2: steeping washed and peeled cassava roots in hot water for 2 to 3 days before drying over the fireplace for 4 to 6 days. 3: steeping washed and peeled cassava roots in hot water for 2 days using ferments before drying under sunlight for 2 days. [c]Storage facilities: see text for description; [d] Storage duration: as estimated by farmers.

Aspergillus spp. were transferred to PDA, CZ (CzapeckDox Agar) and MEA (Malt Extract Agar)at 25°C for 5 days following Raper and Thom (1949), Raper and Fennell (1965) and Singh et al. (1991). Putative *Fusarium* spp. were later transferred to PDA and MEA at 20 and 25°C for 5 days, according to the synoptic keys of Nelson et al. (1983) and Singh et al. (1991). The other fungal species were cultured on PDA and incubated at 25°C for 5 days for further identifications, according to the general keys of Barnet and Hunter (1972).

Colony identifications of plates with pure cultures were made under a compound microscope mounted with a photographic apparatus. Pure cultures of *Penicillium* and *Aspergillus* spp. were sent to the Technical University of Denmark for speciation, whereas *Fusarium* spp. were sent to the Program on Mycotoxin and Experimental Carcinogenesis (PROMEC) in South Africa for identification confirmation.

Data analysis

The proportion of pieces of cassava chips within samples contaminated by the fungi was assessed after the cultures had been examined. The total number of samples contaminated (Nsc) by each species and the total number of isolates (Tnil) of individual fungal species recovered per location were recorded. The relative index frequency (RIF) of each fungal species was calculated as the ratio of the number of isolates of individual fungal species obtained from each sample over the total number of all fungal isolates recovered from chips of each replicate, and expressed as percentage. To increase normality, percentage data (X) were transformed to the Arc sin using the function Y = 180/3.14 × Arc sin (X/100)1/2. These were further subjected to analysis of variance using the General Linear Model (GLM) procedure (Gomez and Gomez, 1984; Anonymous, 1997) of SAS (SAS Institute, Inc., Cary, NC, USA, version 9.2). Using moisture content, percentage recovery of individual fungal species in samples and level of insect infestation of samples as class variables, the relationship between the above mentioned parameters and the susceptibility of chips to fungal contamination could hence be assessed.

To determine the locality with the greatest diversity of isolates, diversity indices were calculated for the various species belonging to each group or category of fungi. The diversity index (DI) was calculated using Simpson's index (Simpson, 1949) as follows:

DI = 1 – Simpson's index:

$$\text{With Simpson's index} = \frac{\Sigma \text{ Total number of isolates of a given species within a fungal species}}{\text{Total number of isolates belonging to all fungal genera identified}}$$

It describes both species richness and species equitability. Simpson's index values may range from 1/S to 1, with S being the total number of species recovered. If every isolate belonging to each fungal genus was a different species, Simpson's index would equal 1/S and be very small, resulting in the maximum diversity index. If all isolates were the same species, the index would be equal to 1, resulting in a minimum diversity index.

RESULTS

Mycoflora isolation

Seventy-three (73) fungal species in 34 genera were isolated from the two forms of cassava chips sampled throughout the three research sites. These included saprophytic and toxigenic fungal species as well as presumptive pathogenic species. In order of importance, *Aspergillus, Fusarium* and *Penicillium,* were the most common genera of fungi recovered from all samples. Other important species or genera of fungi isolated include *Acremonium* sp.*, Armillariamellea* (Vahl& Fr.) *Kummer, Botryodiplodia theobromae Pat., Cladosporium* spp., *Colletotricum* spp., *Corticium rolfsii Curzi, Curvularia* spp., *Fomes lignosius* (Fr.) Fr., *Geotricum candidum, Phythophthora* spp., *Pythium* spp., *Ryzopus nigricans, Thamnidium elegans* and *Trichoderma* spp. However, as a result of their involvement in mycotoxicological processes, only *Aspergillus, Fusarium, Penicillium* sp. are of concern in this paper.

The number of samples contaminated (Nsc), the total number of fungal isolates (Tnil) obtained for each species, and the percentage (RIF) of each species within each fungal genus are given and presented for each location (Tables 2, 3 and 4).

The genus *Aspergillus* with 1827 isolates was the most important group of fungi isolated (Table 2). It accounted for over 57% of the total fungal population, with the largest number of isolates in Mbalmayo (620) and the lowest in Ebolowa (594) (Table 2, 3 and 4). The *Aspergilli* included 14 species altogether and were most numerously represented by the *Aspergillus flavi* group (35% of all *Aspergillus* isolates), others include *Aspergillus tamarii*, and three other aflatoxin-producing species (*Aspergillus parasiticus, Aspergillus nomius* and *A. flavus*).

Within this group, *A. tamarii* was cumulatively isolated from 21 samples in Mbalmayo and Yaoundé, but from only eight samples in Ebolowa. Among members in the aflatoxin-producing species, *A. flavus* was the most commonly encountered species. It represented over 23% of all *Aspergillus* isolates recovered. This species contaminated more samples in Yaoundé (73.8% of samples) than Mbalmayo and Ebolowa. In these two locations, no significant variation in the levels of samples contaminated (P<0.05) by isolates of this species was observed (Table 2). Two additional aflatoxin-producing species, *A. nomius*, and *A. parasiticus* also occurred, but at lower rates (Table 2).

Aspergillus clavatus was the second most important *Aspergillus* species isolated. It was found in all blocks and recovered from 71 out of 132 samples. Its incidence was lower in Yaoundé (75 isolates in 17 samples) and Mbalmayo (93 isolates in 17 samples) than in Ebolowa where 236 isolates were found contaminating 37 samples.

A. niger contaminated over 50% of the sampled chips. Its incidence was more important in Mbalmayo where 131 isolates were obtained in 24 samples. Despite its low level of occurrence in Ebolowa (22 isolates in 4 samples), there was no significant difference (P< 0.05) in the incidence of *Aspergillus aculeatus* in Yaoundé (51 isolates in 12 samples) and Mbalmayo (56 isolates in 11 samples). The relative frequency index, the number of isolates and samples contaminated are presented in Table 2 for the remaining *Aspergillus* spp.

The genus *Fusarium*, with 9 species identified was found associated with a total of 929 isolates. With over 32% of the total fungal isolates, *Fusarium oxysporum* was the most commonly isolated species in this group. Its incidence was greater in Yaoundé (more than 100 isolates in 26 samples) and Mbalmayo (more than 100 in 20 samples).

Fusarium semitectum, although not equally abundant in all locations was the second most important *Fusarium* sp. It was isolated in some samples collected from each of the three sites. It accounted for over 22% of the total *Fusarium* isolates, with the greatest expression rate in

Table 2. Occurrence[a] and incidence of *Aspergillus* sp. on stored cassava chips.

Species	Yaoundé			Mbalmayo			Ebolowa			Overall		
	Nsc	Tnil	RIF	Nsc	Tnil	RIF	Nsc	Tnil	RIF	Nsc	Tnil	RIF
Aspergillus sp.												
A. aculeatus Liz.	12	51	8.3	11	56	9.0	4	22	3.9	27	129	7.06
A. candidus Link	5	16	2.6	3	16	2.6	3	8	1.4	11	40	2.19
A. clavatus Des.	17	75	12.2	17	93	15.0	37	236	41	71	404	22.1
A. flavipes Th. & Cu.	11	40	6.5	15	62	10.0	3	18	3.2	29	120	6.57
A. flavus Link	31	159	25.5	18	135	21.8	19	127	22	68	421	23.0
*A. fumigatus*Fres.	16	37	6.0	4	14	2.3	6	13	2.3	26	64	3.50
A. niger Van Thi..	20	77	12.6	24	131	21.1	9	49	8.6	53	257	14.1
A. nomius Kurtz.	11	29	4.7	3	17	2.7	2	2	0.4	16	48	2.63
A. ochraceus Wilh.	12	32	5.2	6	34	5.5	3	13	2.3	21	79	4.32
A. parasiticus Speare	8	19	3.1	2	6	1.0	0	0	0.0	10	25	1.37
A. sydowii Th. & Cu.	3	3	0.5	0	0	0.0	14	52	9.1	17	55	3.01
A. tamarii Kita	21	68	11.1	21	56	9.0	8	32	5.6	50	156	8.54
A. terreus Thom	1	3	0.5	0	0	0.0	0	0	0.0	1	3	0.16
*A. versicolor*Tir.	2	4	0.7	0	0	0.0	4	22	3.9	6	26	1.42
Total number of isolates		613			620			594			1827	

[a] Data were back transformed after analysis of variance; Nsc: Number of samples contaminated; Tnil: Total number of fungal isolates associated with each species per location; RIF: Relative index frequency of the fungus: This parameter was calculated as the ratio of the number of cassava chips pieces with visible contamination by each fungus over the total number of fungal isolates obtained per location for the corresponding fungus: that is 613 isolates in Yaoundé, 620 in Mbalmayo and 594 in Ebolowa.

Table 3. Occurrence[a] and incidence of *Fusarium* sp. on stored cassava chips.

Species	Yaoundé			Mbalmayo			Ebolowa			Overall		
	Nsc	Tnil	RIF	Nsc	Tnil	RIF	Nsc	Tnil	RIF	Nsc	Tnil	RIF
Fusarium sp.												
F. chlamydosporum W	2	4	1.44	4	11	4.60	7	28	6.8	13	43	4.63
*F. equiseti*Sacc.	12	19	6.83	6	18	7.53	12	44	11	30	81	8.72
*F. verticillioides*Nir.	16	40	14.39	5	28	11.72	12	48	12	33	116	12.49
F. nelsonii Mar.et al.	0	0	0.00	2	7	2.93	3	7	1.7	5	14	1.51
F. oxysporum Schl.	26	105	37.77	20	103	43.10	18	90	22	64	298	32.08
F. sambucinum Fuckel	2	3	1.08	1	6	2.51	10	40	9.7	13	49	5.27
F. semitectum Be. & R	22	63	22.66	5	36	15.06	19	106	26	26	205	22.07
F. solani (Mart.) Sacc.	21	44	15.83	3	23	9.62	12	35	8.5	36	102	10.98
F. stilboides Wollenw.	0	0	0.00	1	7	2.93	1	14	3.4	2	21	2.26
Total number of isolates		278			239			412			929	

a: Data were back transformed after analysis of variance; Nsc: Number of samples contaminated; Tnil: Total number of fungal isolates associated with each species per location; RIF: Relative index frequency of the fungus: This parameter was calculated as the ratio of the number of cassava chips piecescontaminated by each fungus over the total number of isolates obtained per location for the corresponding fungus: that is 278 isolates in Yaoundé, 239 in Mbalmayo and 412 in Ebolowa.

Ebolowa (25.7% of isolates) and the lowest in Mbalmayo (15%).

Fusarium verticillioides (formerly *Fusarium moniliforme*), a *fumonisin*-producing fungus, contaminated 38% of samples in Yaoundé, 11% in Mbalmayo, and almost 27% of samples collected at Ebolowa. This species which formed more than 12% of the total fungal *Fusarium* isolates, was the third most important *Fusarium* spp.

Fusarium chlamydosporum, Fusarium equiseti, and *Fusarium sambucinum* were also isolated and occurred in all the locations surveyed. *Fusarium nelsonii* and *Fusarium stilboides* were rarely recovered from the samples analyzed. Their incidence was only observed in

Table 4. Occurrence[a] and incidence of *Penicillium* sp. on stored cassava chips.

Species	Yaoundé			Mbalmayo			Ebolowa			Overall		
	Nsc	Tnil	RIF	Nsc	Tnil	RIF	Nsc	Tnil	RIF	Nsc	Tnil	RIF
Penicillium sp.												
P. aethiopicum Thom	1	4	3.77	2	4	3.03	0	0	0.0	3	8	1.79
P. brazilianum Thom	0	0	0.00	0	0	0.00	3	9	4.3	3	9	2.01
P. citrinum Thom	25	59	55.66	22	72	54.55	31	104	50.0	78	235	52.46
P. duclauxii Delacroix	5	13	12.3	6	26	20	0	0	0.0	11	39	8.71
*P. herquei*Bain& Sart	1	2	1.89	3	6	4.55	6	32	15.0	10	40	8.93
P. minioluteum Dierc.	2	3	2.83	3	5	3.79	1	6	2.9	6	14	3.13
P. oxalicum Cu.& Th.	0	0	0.00	1	1	0.76	0	0	0.0	1	1	0.22
P. paneum Boysen	6	12	11.32	2	3	2.27	0	0	0.0	8	15	3.35
P. paxillii Bainier	3	6	5.66	2	3	2.27	1	3	1.4	6	12	2.68
P. piceum Ra.& Fen.	0	0	0.00	1	3	2.27	0	0	0.0	1	3	0.67
P. purpurogenum Th.	4	7	6.60	2	3	2.27	10	14	6.7	16	24	5.36
P.sclerotiorum van Be.	0	0	0.00	0	0	0.00	4	18	8.6	4	18	4.02
*P. sumatrense*Szilv.	0	0	0.00	2	6	4.55	0	0	0.0	2	6	1.34
*P. steckii*Zaleski	0	0	0.00	0	0	0.00	11	24	11	11	24	5.36
Total number of isolates		106			132			210			448	

a: Data were back transformed after analysis of variance; Nsc: Number of samples contaminated; Tnil: Total number of fungal isolates associated with each species per location; RIF: Relative index frequency of the fungus: This parameter was calculated as the ratio of the number of cassava chips pieces contaminated by each fungus over the total number of isolates obtained per location for the corresponding fungus: that is 106 isolates in Yaoundé, 132 in Mbalmayo and 210 in Ebolowa.

Mbalmayo and Ebolowa where they contaminated a very low proportion of the samples examined (Table 3).

Within the genus *Penicillium*, 448 isolates representing 14 species were associated with the samples analyzed. Their relative importance varied and Mbalmayo was the location associated with the largest number of *Penicillium* spp. (Table 4).

Penicillium citrinum, Penicillium herquei, Penicillium paneum, Penicillium paxillii and *Penicillium purpurogenum* were found contaminating chips of all locations in some samples. The most commonly isolated species was *P. citrinum*, for which 235 isolates or 52% of all *Penicillium* spp. were found contaminating almost 60% of all samples analyzed. *Penicillium duclauxii* was not found on samples collected from Ebolowa. Isolates of this species along with those of *P. herquei* were hosted by almost an equal number of samples. In this respect, ten samples were associated with the presence of *Penicillium duclauxii*, whereas eleven samples were colonized by isolates of *P. herquei*. An important observation associated with *Penicillium* species occurrence is that *P. herquei* was less abundant where the incidence of *P. duclauxii* was higher (Table 4).

Six other species of *Penicillium* with very low incidence were observed to occur in only one location. These were *Penicillium stecki, Penicillium sumatrense, Penicillium oxalicum, Penicillium sclerotiorum, Penicillium piceum* and *Penicillium brazilianum*. The total number of isolates associated with each never exceeded 20, and these could be considered minor components of species belonging to this genus.

Diversity of fungi isolated and relationships between contamination of chips by fungi and the parameters studied

The diversity indices associated with the fungal groups of concern in this study were computed, and varied between 0.65 and 0.85 depending on locations, and fungal genera (Table 5). The values obtained for each location suggest that Yaoundé and Mbalmayo were sources of greater fungal species diversity. From a generic point of view, *Aspergillus* spp. yielded the largest diversity indices except in Ebolowa. On the other hand, *Fusarium* and *Penicillium* spp. were connected with more diverse populations at Ebolowa, when compared with Yaoundé and Mbalmayo.

Six stored-product insects were found infesting some samples in the course of this study. The taxa detected were *Araecerus fasciculatus De Geer (Coleoptera: Anthribidae), Dinoderusbifovealatus Wollaston (Coleoptera: Bostrichidae) Carpophilus dimidiatus F. (Coleoptera: Nitidulidae) Carpophilus hemipterus L. (Coleoptera: Nitidulidae) Periplaneta americana L. (Dictyoptera: Blatidae),* and *Sitophilis zeamais* Motschulsky *(Coleoptera: Curculionidae).* The level of infestation varied between 1 and 20 insects per 100 g of cassava chips in some samples (data not shown). At the same time, the moisture content associated with the

Table 5. Diversity indices associated with *Aspergillus*, *Fusarium* and *Penicillium* spp. isolated from stored cassava chips in three locations of southern Cameroon.

Species	Yaoundé	Mbalmayo	Ebolowa	Total[1] genus
Aspergillus spp.	0.85	0.77	0.68	0.83
Fusarium spp.	0.76	0.76	0.84	0.79
Penicillium spp.	0.65	0.66	0.70	0.68
Total mycoflora	0.81	0.81	0.78	

Diversity index calculated using Simpson's index. [1]Figures for total represent a separate calculation rather than an average for each location or each fungal genus.

Table 6. Analysis of variance of the relationship between the susceptibility of chips to fungal contamination and the incidence of location of sample collection, chip type, moisture content, levels of insect infestation of individual fungal species.

Source of variation	Degrees of freedom	Means squares	F values	Pr (F)
Location	2	40.0068	3.91	0.0201
Chip type	1	5.747	0.56	0.5702
Moisture content	127	16.549	1.61	0.0001
Insect infestation	5	8.982	0.88	0.5953
Fungal species	35	347.29	33.94	0.0001

samples collected ranged from 4.3 to 29.3% and averaged 12.6% (data not shown). These two parameters (moisture content and level of infestation of samples by insects), along with the type of chips, the incidence of individual fungal species, and the location of sample collection were assessed for their effect on the susceptibility of cassava chips for fungal contamination.

According to information summarized in Table 6, the level of insect infestation, and the type of chips had no significant effect on the level of contamination of cassava chips by fungi ($P<0.05$). The moisture content ($P< 0.0001$), the locations of sample collection, and the species of fungi ($P<0.0001$) led to significant different results. This shows that variations in moisture content values and possible differences in environmental factors across the locations surveyed allowed the contamination of cassava chips by different species of fungi.

DISCUSSION

Many fungi have been reported as contaminants of stored food products worldwide. Accordingly, the association of 73 fungal species with stored cassava chips was not unexpected. Our study however provides the first documentation on the distribution and frequency of occurrence of these deteriogens on stored cassava chips in 45 villages of southern Cameroon. The results obtained suggest that species in the genera *Aspergillus*, *Fusarium* and *Penicillium* are fungi commonly associated with stored cassava chips.

Among fungi in the genus *Fusarium, F. stilboides* and *F. nelsonii* were inconsistently isolated from chips. These species were reported as commonly associated with plant debris and soil samples throughout the world (Marasas et al., 1998). The rest of the *Fusarium* spp. formed part of the mycoflora recovered from transformed cassava products during similar surveys in Benin and Nigeria (Msikita et al., 2001). In this study, *Fusarium* spp. were present in all blocks surveyed but not equally abundant. *F. oxysporum* formed the greatest proportion of this complex. Its incidence increased as that of other *Fusarium* spp. decreased. Similarly, *F. verticillioides* was isolated with highest frequencies where the incidence of *F. solani*, and *F. equiseti* was less important (Table 3). Available arguments accounting for these configurations between species are somewhat limited. However, this could tentatively be explained by a possible competition between species (Kommedhal et al., 1979; Essono, 2008).

In all respects, *Aspergillus* and *Penicillium* spp. obviously constituted the predominant species of the mycoflora associated with cassava chips in storage. These represented over 71% of the species belonging to all three fungal genera. The present results are consistent with data on wheat obtained by Pehlate (1968), on cotton seeds by Mazen et al. (1990), and on tiger nuts by Bankole and Eseigbe (1996) who reported these two genera to be the major components of the mycoflora of stored food products during similar investigations.

In a monographic study, Frisvad and Samson (1991) reported species in the genus *Penicillium* to be more common in temperate areas, and mainly associated with cereal-based foods in storage. Evidence for this ecological specialization was not ascertained from the present study. Instead, besides the frequent isolation of *P. citrinum*, known for its high prevalence in subtropical and tropical zones (Raper and Thom, 1949), thirteen additional *Penicillium* spp. were recovered from our samples and from all the locations investigated, indicating that *Penicillium* spp. might have a distribution not only in colder ecologies, but also in warm tropical zones and other food products. Their inconsistent recovery when compared with *Aspergillus* species, may be because species in this genus do not grow well at the high temperatures that usually characterize tropical areas (Hussaini et al., 2009).

The recovery of *A. flavus* in 68 samples from 36 out of 45 villages surveyed suggests that this fungus is a common contaminant of stored cassava chips. The mean incidence associated with its level of occurrence in the three locations, however, varied greatly. Climatic factors alone such as temperature, rainfall, and relative humidity as suggested in some studies (Doster and Michailides, 1994; Viquez et al., 1994) were not found adequate to explain the fluctuations observed in the incidence of this species among the locations surveyed. In this study, the incidence of *A. flavus* varied in an inverse relationship with high rainfall and temperatures across the benchmark, according to general data associated with these climatic parameters in the area investigated (Anonymous, 1996).

This relationship was reflected by the high percentage contamination means of the fungus in Yaoundé samples where mean values of these climatic factors were low as compared to Ebolowa. It is highly probable that processing practices and storage systems in use in those locations were responsible for the observed variations. To this effect, cassava balls from Ebolowa, hung above a fireplace, were associated with the lowest number of isolates (Table 2). Accordingly, it could be postulated that the temperatures prevailing under those storage facilities in which balls are preserved were presumably higher than the 25°C optimum observed for growth of *A. flavus* (Essono et al., 2007). This would have prevented this species from developing intensively in samples originating from Ebolowa which were preferably stored under such conditions.

The diversity indices associated with the three fungal genera dealt with in this study greatly varied both between fungal genera, and locations. The high degree of variation observed in their values among fungal groups could be attributed to differences in practices implemented during chips production, or more importantly, to the mode of dispersal and survival of species associated with the fungal genera of concern in this study. The similarity in species diversity observed between locations in Mbalmayo and Yaoundé may be because these two areas share the same phytogeographical characteristics (Anonymous, 1996). In addition, surveys aimed at collecting information related to processing and constraints aspects of cassava transformation showed that farmers in Yaoundé and Mbalmayo processed and stored cassava chips in the same way, different from that in Ebolowa (Essono et al., 2008).

The main objectives of this study were to survey and determine the mycoflora complex associated with stored cassava chips traditionally produced in southern Cameroon by rural farmers, and to note the prevalence of fungi in the genera *Aspergillus, Fusarium,* and *Penicillium.* The results obtained showed that toxigenic *Aspergillus* spp. were the most prevalent fungi contaminating stored cassava chips. They accounted for over 57% of the total fungal isolates belonging to the three genera of concern in this study. From the present results, there is clear evidence that colonization of cassava chips by all the toxigenic fungi recovered from the samples analysed may have serious consequences for human and animal health as a result of their contamination by their respective mycotoxins. This contamination is reported to be most acute and widespread in the warm and humid areas of Africa, Asia and Latin America, where adequate conditions exist for their subsequent development (CAST, 2003; Williams et al., 2004). As the rate and degree of mycotoxin contamination are often believed to be dependent on a number of factors among which are the moisture content and storage conditions such as damage by insects (Turner et al, 2005), farmers should be trained on how to avoid insect spoilage of stored cassava products used as food or feed so as to limit their infestation by toxigenic fungi. During household surveys, visible insect damages were observed and mainly characterized by holes created on cassava chips. It could therefore be hypothesized that propagules' transmission of mycotoxin-producing fungi is likely, since insect damages are often viewed as access routes for toxigenic fungi penetration in food and feed products while in storage (Hell et al., 2008).

In all respects, because the most taxa detected in the present study are known for their potential capability to produce mycotoxins, the results obtained may provide guidance for better orienting and targeting mycotoxin research on stored cassava products.

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Prevalence and antimicrobial resistance profile of *Salmonella* isolates from dairy products in Addis Ababa, Ethiopia

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A cross sectional study was conducted on dairy items in Addis Ababa from October 2010 to March 2011 to determine prevalence and antimicrobial resistance profile of *Salmonella*. A total of 384 dairy items, 96 of each item (cheese, milk, butter and yogurt) was sampled. The overall prevalence of *Salmonella* was found to be 1.6% (6 of 384). Prevalence of 3.1, 1.04, 2.1, and 0% was observed from cheese, butter, milk and yogurt, respectively. However, there was no statistically significant difference (P > 0.05) in the prevalence of *Salmonella* among the different sample types. Isolates were tested for the effects of eight antimicrobials by disk diffusion technique; all isolates were resistant to one or more of the tested antimicrobials. Of all isolates, 50% were multiple antimicrobial resistant. 83.3, 50, 16.7, and 16.7% of isolates were resistant to tetracycline, ampicillin, amoxicillin, and chloramphenicol, respectively. However, all the isolates were susceptible to gentamycin, ceftriaxone, ciprofloxacin, and sulfamethoxazole. From this pilot study, we concluded that dairy products are a potential source of *Salmonella* infection with antimicrobial resistance. Furthermore, hygienic management of dairy products and prudent use of antimicrobials are also suggested.

Key words: Salmonella, prevalence, dairy products, antimicrobial resistance, Addis Ababa.

INTRODUCTION

Salmonella is a leading cause of food borne illness (WHO, 1988; White et al., 2001). Globally, more than 93 million cases of gastroenteritis are caused by non typhoidal *Salmonella* with 155,000 deaths each year. Of these cases, 80.3 million cases were estimated to be food borne. Salmonellosis, the diseases caused by bacteria of the genus *Salmonella*, is a common intestinal illness caused by numerous *Salmonella* serovars with clinical manifestations that vary from severe enteric fever to mild food poisoning (Jones et al., 2004) both in animals (Radostits et al., 2007) and humans (Hohmann, 2001). Foods of animal origin particularly meat, poultry, egg, milk and milk products are considered to be the primary source of human salmonellosis (Acha and Szyfers, 2001). Most of these food products become contaminated during slaughter, processing in contaminated environment and because of faulty in transport, handling, storage or preparation.

Salmonellosis takes a healthy tool in human life and suffering, particularly among infants and children, the elderly and other susceptible persons particularly in developing countries where most food industries are not

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well aware of food safety issues and knowledge of modern technologies (Van der Venter, 1999). Good Manufacturing Practices, hygiene, Hazard Analysis Critical Control Point systems and quality control are often limited or absent in such countries. Cold storage facilities are inadequate and quality of water used for food processing may not be suitable. The vast numbers of labor that handle food in factories, as well as on farms are illiterate and untrained. In such countries lack of information leads to lack of appreciation of health significance of unsafe food (Van der Venter, 1999).

Salmonellosis has a wide variety of domestic and wild animal hosts (Acha and Szyfers, 2001). Infection in animal is of importance because of the direct economic consequences of salmonellosis attributable to mortality and morbidity. Of even greater importance are the human health consequences of salmonellosis acquired by direct or indirect contact with animals, which constitute a vast reservoir of these organisms (Libby et al., 2004). Several species of *Salmonella* have been documented to colonize udder and shed at level of 2000 organisms per milliliter of milk (Fontaine et al., 1980).

According to Jayaroo and Henning (2001) *Salmonella* was isolated from 6.1% of bulk tank milk sample from dairy herds in South Eastern Dakata and Western Minnesota. Cheeses made from raw milk have been implicated as sources of several outbreaks (D'Aoust, 1994). The worst food poisoning incident due to *Salmonella* occurred in US in 1985 and there was a cause of 16,289 human cases and 7 deaths as the result of recontamination of pasteurized milk with a potent strain of *Salmonella* Typhimurium. In 1994, there was also national outbreak of *Salmonella* Enteritidis affecting 225,000 people who consumed contaminated ice cream products (Doyle and Cliver, 1990). In *Salmonella*, in addition to concern about the presence of it as a potential food borne pathogen, concern has also been raised about the human health impact of presence of antimicrobial resistance transferred among these organisms (Dargatz et al., 2003), which limits therapeutic options for treatment of disease in human and animals. Studies show that antimicrobial resistance *Salmonella* are increasing due to the use of antimicrobial agents in food animals, which are subsequently transmitted to humans usually through the food supply (White et al., 2001).

In Ethiopia, despite attempts to study prevalence of *Salmonella* mainly in poultry and beef, the status in milk and milk products is still unknown. However, studies made elsewhere indicated that milk and milk products are important source of *Salmonella* particularly among those raw consumers (WHO, 1988; Jay, 2000). Ubiquitous nature of *Salmonella,* unhygienic condition prevailing at the farm levels and food handlers, and habit of consuming milk and milk products in raw suggest that milk and milk products can act as source of *Salmonella* organisms in Ethiopia. Considerable proportion of them might have developed resistance to antimicrobials that are commonly used in both the veterinary and public health. Such a problem might be significant in areas like Addis Ababa where consumption of milk and milk products are high, dairy supermarkets are significant, and handling of milk and milk products take several hours until they reach to consumers. Therefore, this study was carried out to estimate the prevalence of non typhoidal *Salmonella* and to determine the antimicrobial resistance profile of *Salmonella* isolates from dairy products in Addis Ababa.

MATERIALS AND METHODS

Study area and sample size

The study was a cross-sectional study conducted in dairy products (butter, cheese, milk and yogurt) which were purchased from different dairy supermarkets of Addis Ababa. The variable of interest considered as an output variable versus risk factors was *Salmonella* status of dairy items. Types and handling of the dairy items were considered as explanatory variables. List of all 81 currently operational supermarkets in Addis Ababa were collected from Addis Ababa municipality. Each of 45 supermarkets was randomly selected using simple random sampling technique and identified for the study. The sample size required for the study was determined based on sample size determination in random sampling for infinite population using expected prevalence of salmonellosis and the desired absolute precision according to Thrusfield (2005). Accordingly, a total of 384 dairy product samples were collected for this study.

Sampling procedure

A total of 384 samples including butter, cheese, milk and yogurt (each n = 96) was randomly purchased from 45 supermarkets. Milk and yogurt samples were collected in separate sterile bottles of 25 ml capacity while cheese and butter samples were collected in sterile plastic bags. Samples were properly labeled by sample type, name of supermarket and date of sample collection and transported in ice box to microbiology laboratory of Aklilu Lemma Institute of Pathobiology for analysis. Upon arrival, the samples were immediately processed or stored over night in a refrigerator at 4ºC until they had been processed in the following day.

Isolation and identification of *Salmonella* organisms

The isolation of *Salmonella* was performed according to the standard operating procedure set by the Global *Salmonella* Surveillance and laboratory support project of the World Health Organization (WHO) and the National Health Services for Wales (NHS), in which both procedures use ISO-6579 (ISO, 2002) Standard for the isolation of *Salmonella*.

Biochemical tests: Pure cultures obtained from nutrient agar were tested biochemically according to ISO 6579 (2002) (ISO, 2002).

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing of the isolates was performed according to the National Committee for Clinical Laboratory Standards (NCCLS, 1999). From each isolate, four to five biochemically confirmed well-isolated colonies grown on nutrient agar were transferred into tubes containing 5 ml of Tryptone soya broth (Oxoid, England). The broth culture was incubated at 35ºC for 4 h

Table 1. Prevalence of *Salmonella* in dairy items in Addis Ababa dairy supermarkets.

Sample type	Number of samples examined	Positive (%)
Cheese	96	3 (3.1)
Yogurt	96	-
Butter	96	1 (1.04)
Milk	96	2 (2.1)
Total	384	6 (1.6)

Fisher's exact = 0.525.

Table 2. Antimicrobial sensitivity test results of *Salmonella* isolates from dairy items of Addis Ababa supermarket.

Type of antimicrobial	Number of isolates			Total
	Resistant (%)	Intermediate (%)	Susceptible (%)	
Gentamycin (10 µg)	-	-	6 (100)	6
Tetracycline (30 µg)	5 (83.3)	1 (16.7)	-	6
Ceftriaxone (30 µg)	-	-	6 (100)	6
Ciprofloxacin (5 µg)	-	-	6 (100)	6
Ampicillin (10 µg)	3 (50)	1 (16.7)	2 (33.3)	6
Amoxicillin (10 µg)	1 (16.7)	1 (16.7)	4 (66.7)	6
Sulfamethoxazole (25 µg)	-	-	6 (100)	6
Chloramphenicol (30 µg)	1 (16.7)	-	5 (83.3)	6
Total	10 (20.8)	3 (6.3)	35 (72.9)	48

All of the total isolates were resistant to one or more of the tested antimicrobials. Of all isolates half (50%) were multiple antimicrobial resistant while the rest half were resistant to single antimicrobial as shown in Table 3. Three types of resistance patterns were observed, resistance to one, two and three antimicrobials as shown in Table 3.

until it achieved the 0.5 McFarland turbidity standard. Sterile cotton swab was dipped into the suspension and the bacteria were swabbed uniformly over the surface of Muller Hinton agar plate (Oxiod CM 0337 Basingstoke, England). The plates were held at room temperature for 30 min to allow drying. Antibiotic discs with known concentration of antimicrobials were placed and the plates were incubated for 24 h at 37ºC. *Escherichia coli* ATCC 25922 was employed as strain of quality control. Each isolate tested for a series of eight antimicrobials. The diameters of zone of inhibition were recorded to the nearest millimeter and classified as resistant, intermediate, or susceptible according to published interpretive chart (NCCLS, 1999)

Data management and analysis

The data were entered and managed in MS Excel work sheet. The analysis was conducted using Intercooled Stata 7. The significance differences between the prevalence of *Salmonella* species in various sample types was determined using Fisher's exact test as the numbers within categories were too small for the Chi-square test.

RESULTS

Of the total 384 cheese, butter, yogurt and milk samples (each n=96), 1.6% (6 of 384) were culture positive for *Salmonella* (Table 1). However, there was no significant difference between the prevalence in the different types of products (Fisher's exact = 0.525, P > 0.05).

Antimicrobial resistance

Of all isolates, 50% were multiple antimicrobial resistant and 83.3, 50, 16.7, and 16.7% of isolates were resistant to tetracycline, ampicillin, amoxicillin, and chloramphenicol, respectively. All isolates were susceptible to gentamycin, ceftriaxone, ciprofloxacin and sulfamethoxazole. The highest level of resistance was observed for tetracycline (83.3%) as shown in Table 2.

DISCUSSION

Prevalence of *Salmonella* in dairy items

In this cross-sectional study on prevalence and antimicrobial resistance of *Salmonella* isolates from dairy products in Addis Ababa, overall 1.6% (6 of 384) *Salmonella* prevalence was detected. *Salmonella* was detected from cheese, butter, and milk with prevalence of 3(3.1%), 1(1.04%), and 2 (2.1%), respectively. However, there was no statistical significant difference in prevalence of *Salmonella* among the different dairy samples (Fisher's exact = 0.525, p > 0.05). Prevalence of *Salmonella* detected from cheese in this study was in agreement with the work of Zewdu (2004) who reported *Salmonella* from cheese with prevalence of 2.1% in his previous study of

Table 3. Antimicrobial resistance patterns of *Salmonella* isolates.

Sample type	Isolate	Antimicrobial resistance pattern
Butter	B	Amp, Caf
Cheese	C1	Te, Amp, Amo
	C2	Te
	C3	Te
Milk	M1	Te
	M2	Te, Amp

Note: B is the one isolate from butter; C1, C2 and C3 are isolates from cheese; M1 and M2 are isolates from milk. Amp is ampicilin; Te is tetracycline; Caf is Chloramphenicol; Amo is amoxicillin.

Salmonella organisms in Addis Ababa. *Salmonella* was detected from milk with prevalence of 2.1%. This result is in agreement with the work of Rohrbach et al. (1992) who reported *Salmonella* in milk with prevalence of 2.68% in France. In addition, Jayaroo and Henning (2001) found high prevalence of *Salmonella* 6.1% in bulk tank milk from South Eastern Dakata and Western Minnesota. The difference in prevalence between this study from that of the study in Western Minnesota might be associated difference in hygienic and farm management practices in the different study areas. According to Radostits et al. (1994) epidemiological patterns of *Salmonella* differ greatly between geographical areas depending on climate, population density, land use, farming practice, food harvesting and processing technologies and consumer habits. However, mentioning the difference in method of detection and variation in sample size as a reason for difference in prevalence cannot be overemphasized.

In the present study, *Salmonella* was detected in a relatively quite low prevalence compared to prevalence detected from other food items and dairy products might not pose a significant health risks to humans. According to D'Aoust (1997) even such level of *Salmonella* might be enough to cause salmonellosis in risk group individuals; newborns, infants, the elderly and immune compromised individuals who are particularly more susceptible to *Salmonella* infections at a lower infective dose than healthy adults. However, it is generally accepted that the presence of any *Salmonella* isolate in a food should be regarded as a potential hazard to human (Fathi et al., 1994). Therefore, even if the study indicated low prevalence of *Salmonella* in dairy products, it is a potential hazard for *Salmonella* infection through consumption of dairy products; which is especially important in Ethiopia in general and Addis Ababa in particular where dairy products are in most of the time consumed without appropriate cooking practices.

Salmonella was not detected from yogurt samples. Yogurt or ''Ergo'' is a traditional Ethiopian fermented milk produced by spontaneous fermentation using traditional utensil. The low prevalence of *Salmonella* in milk to prepare the yogurt as indicated in this study might contribute for absence of *Salmonella* in yogurt samples. Partly, it might also be associated the relatively hygienic practice exercised during preparation and marketing of yogurt and due to low pH (Makita et al., 2012). *Salmonella* are destroyed or inactivated during the fermentation of high acid products such as yogurt in which pH value is less than 4.55 (Varnman and Evans, 1991). In Ethiopia, there is a habit of smoking of utensils which are used for preparation of yogurt. This was scientifically justified by Mogessie and Fekadu (1993) and Lemma (2004) that smoking reduces the undesirable microbial contamination which enhances the fate of fermentation and passing the smoke flavor to the milk; this might also contribute for absence of detection of *Salmonella* from yogurt samples. However, the prevalence of *Salmonella* in yogurt in this study cannot enable to disregard yogurt as a vehicle for salmonella infection.

Antimicrobial resistance

Antimicrobial resistant *Salmonella* isolates to commonly used antimicrobials were detected; all isolates were resistant at least for one antimicrobial. Of the total tested isolates; 83.3, 50, 16.7, and 16.7% of isolates were resistant to tetracycline, ampicillin, amoxicillin and chloramphenicol, respectively. However, all the isolates were susceptible to gentamycin, ceftriaxone, ciprofloxacin, and sulfamethoxazole. All of the total isolates were resistant to one or more of the tested antimicrobials; 50% were multiple antimicrobial resistant while the rest half were resistant to single antimicrobial. This finding is in contrast to Zewdu (2004) who reported 25% antimicrobial resistant *Salmonella* isolates from cottage cheese. Detection of antimicrobial resistant *Salmonella* might be associated with their frequent usage both in livestock and public health sectors as these antimicrobials are relatively cheaper and commonly available (D'Aoust, 1997). The effectiveness of gentamycin, ceftriaxone, ciprofloxacin, and sulfamethoxazole in this study might be due to the difference in frequency of usage among the available antimicrobials, the nature of drugs, and their interaction with the bacteria. Different individuals reported antimicrobial resistant *Salmonella* isolates in previous studies from Ethiopia (Gedebou and Tassew, 1981; Ashenafi and Gedebou, 1985; Molla et al., 1999; Molla et al., 2003) and from other countries (D'Aoust et al., 1992; White et al., 2001).

The findings of 100% antimicrobial resistant *Salmonella* isolates from examined dairy items samples were remarkable. It represents public health hazard due to the fact that food poisoning outbreaks would be difficult to treat and this pool of MDR *Salmonella* in food supply represents a reservoir for the transferable resistant genes (Diaze De Aguayo et al., 1992). The reasons for the recovery of antimicrobial resistance *Salmonella* isolates was most likely due to the indiscriminate use of antimicrobials (WHO, 1988; Guthrie, 1992), self medication due

to easy access to antibiotics without prescription in public health sector, and administration of sub therapeutic dose of antimicrobials to livestock for prophylactic or nutritional purpose (Acha and Szyfers, 2001). This might also be due to the use of antimicrobials for the promotion of growth and prevention of disease in food animals.

The present study indicated importance of dairy products as potential source of *Salmonella* infection. All the isolates were resistance to one or more of the tested antimicrobials. Tetracycline, ampicillin, amoxicillin, and chloramphenicol are less effective drugs against *Salmonella* isolates which limit therapeutic choice both in animal and human health care management. Therefore, food manufacturers should design comprehensive programs as Good Manufacturing Practice (GMP) and implementation of Hazard Analysis and Critical Control Points (HACCP) system to ensure the freedom of dairy products from this pathogen and concerned individuals should create awareness to food handlers and consumers.

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Full Length Research Paper

Study on prevalence of bovine mastitis in lactating cows and associated risk factors in and around Areka town, Southern of Ethiopia

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A cross-sectional study was conducted from October 2011 to March 2012 on a total of 384 lactating (Zebu 183, Jersey103 and Holstein 93) dairy cows randomly selected from 29 rural kebele in which three small scale dairy farms were included. The study was designed with the objective to determine the prevalence of mastitis and isolate the major bacteria that causes mastitis involved in areka woreda and its surrounding wolayta zone. Milk samples was collected and bacteriological culture was done and further confirmation was done by BIOLOG identification system. The overall prevalence of mastitis in the area was 52.9% (n = 203), out of which 9.4% (n = 36) were clinical and 43.5% (n =167) were sub-clinical cases. Among the isolated bacterial genera, the isolate were *Staphylococcus* (14.8%), *Streptococcus* (7.5%), *Corynebacterium* (0.52%) and *coliform* (0.25%). Characterization was also under taken and the species recovered were *Staphylococcus aureus* 136 (54.4%) dominating followed by *Streptococcus dysagalactiae* 62 (24.8%), *Staphylococcus intermidius* (8.4%) *Streptococcus.uberis* 13 (5.2%) *Staphylococcus epidermides* (4.4%) *Streptococcus agalactia* 4 (1.6%), *Corynebacterium pyogens* 2 (0.8%) and *Escherichia coli* 1 (0.4%). There was no statically significant variation (P>0.05) between breeds and the parity number of the cow, but the prevalence of mastitis was found to be statistically significantly among different age groups and lactation stages (p<0.05). The study shows that mastitis is significant problem of dairy cows in the study area and the major isolated bacteria were contagious pathogens. Therefore, hygienic milking practice, culling of chronically infected cows and hygienic practice in the environment should be followed.

Key words: Bovine, mastitis, prevalence, Areka Woreda, SNNPR, Ethiopia.

INTRODUCTION

Mastitis is complex disease that generally involves interplay between management practices and infectious agents, having different degrees of intensity and variations in duration and residual effects. Various infectious agents numbering more than twenty different groups including bacterial, viruses, yeast, fungi and

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rickettsia with bacterial being the major cause have been reported (Biffa et al., 1999). At least, 137 infectious causes of bovine mastitis are known to date and in large animals, the commonest pathogens are *Staphylococcus aureus, Streptococcus agalactiae,* other *Streptococcus* species and coliforms (Sumathi et al., 2008). It may also be associated with many other organisms including *Actinomyces pyogenes*, *Pseudaomonas aeruginosa*, *Nocardia asteroides*, *Clostridium perfringens* and others like *Mycobacterium*,*Mycoplasma*, *Pastuerella* and *Prototheca* species and yeasts (Rodostits et al., 2007).

In Ethiopia, the incidence and distribution of the disease has not been studied systematically and information relating to economic loss and the overall prevalence of the disease is inadequate. The economic losses due to mastitis that the state farms in Ethiopia are experiencing are not difficult to imagine as more than 10% of cows in most farms have at least one blind quarters (Goshu et al., 1985).

The environmental condi-tion of Areka woreda is assumed to be one of the representatives of mid high lands of Ethiopia. During the rainy seasons, the environment assumes muddy wet, humid and moisture conditions which favors the multi-plication and grown of various microorganisms and potentiate their disease producing capacity. Therefore, the aim of this study was to isolate the major causative agent and to assess the major risk factors responsible for mastitis in the study area.

MATERIALS AND METHODS

Study area

The study was conducted in Areka woreda which is in SNNPR in Wolaita Zone located 360 km from Addis Ababa, the capital of Ethiopia. The area is bounded with Damot Gale Woreda to the East, Damot soria Woreda to the south, Balso bomba Woreda to West and Hady Hadero Woreda to the north. Its altitude ranges from 1650 to 2980 (m.a.s.l). It receives an annual rainfall ranging from 1000 - 1200 mm and an annual temperature range of 25-35°C. The area is categorized under Woina-dega agro-ecological climate.

Study design

Cross-sectional study was conducted from October 2011 to April 2012 in Areka woreda.

Study population

The study populations were lactating cows of different age, lactation stage, parity and breed of some dairy farm and smallholder farms of the town including surrounding Keble. Representative Keble was selected using simple random sampling methods.

Sample size

The sample size was determined according to the formula given by Thrusfield (2005) by taking pervious prevalence of mastitis 50%. Accordingly, the calculated value for sample size is equal to 384. Data regarding the different potential risk factors (age, parity, lactation stage, housing conditions and previous history of mastitis) was collected for 384 lactating cows from farm records when available and by interviewing the farm owner when not available.

Milk sample collection

A milk sample was collected according to the National Mastitis Council (NMC) (1990).

Culture and identification

Bacteriological examination was done according to the National Mastitis Council Guideline (1990). Finally, pure colony was taken and sub-cultured on BUG (BiOLOG Universal Growth Media) at 37°C for 18-24 h as a primary and secondary culture. Well-isolated fresh colonies from BUG (Biolog, USA) media are inoculated into 18-20 inoculation fluid to have bacterial suspension with turbidity equivalent to 20% transmittance as measured by turbidity meter. This suspension was poured into of Micro plates with multi-channel pipettes. The Micro Plates were loaded into Omnilog tray to be incubated, analyzed and interpreted for 18-24 h as per guidelines of BiOLOG Users Guideline (2008) and finally identified bacteria was obtained and printed out.

Statistical analysis

The data collected and recorded on specifically designed forms and prepared for analysis was entered in the Microsoft excel spread sheet and analyzed with SPSS version 16 statistical software. Descriptive statistic was used to summarize the data generated from the study. The prevalence of mastitis (clinical and sub-clinical) was calculated by using percentage values and possible association of disease with risk factors was analyzed by using Chi-square test and predictive value (P-value).

RESULTS

A total of 384 dairy cows were examined for the presence of mastitis both clinically and by the use of screening test, California Mastitis Test (CMT) supported by detailed bacteriological examinations and the results were summarized in the following subtitles. The overall prevalence of the disease was 52.9%.

Prevalence of mastitis by breed

Breed difference can play a vital role in the prevalence of different diseases. In this study area, different breeds of cows are there especially at farm level (Table 1). The pre-

Table 1. The prevalence of mastitis by breed.

Breed	Positive no. (%)	Negative no. (%)
Jersey	54(52.4)	49(47.6)
Zebu	91(49.7)	92(50.3)
Holstein	58(55.9)	40(43)
Total	203(52.9)	181(47.1)

$X^2 = 2.297$, P = 0.309.

prevalence of the three breeds namely: indigenous zebu, Holstein and Jerseys was found statistically not significantly different (P>0.0.5).

Prevalence of mastitis based on lactation stage

With regard to the inflammation of the udder of different causes, the lactation stage plays an important role and it was considered as one risk factor for the prevalence of the mastitis in this study. The difference in the presence of mastitis in the study population according to the category set was statistically significant indicating that those animals at the end stage of lactation were affected more (Table 2).

Prevalence of mastitis based on the number of parity

The prevalence measure based on the number of the parity was statistically not significant. The infection generally increases with increasing lactation number. According to this study, the higher occurrence of infection, 58.3% was in 3rd and 4th parity groups and lower in 5th and 6th parity groups (Table 3).

Prevalence of mastitis based on different age groups

Age is a detrimental factor in the distribution of the diseases because at some time it is stressor. It was taken into consideration and the prevalence of mastitis was measured for different age groups of lactating cows. The prevalence was found to be much higher in the young and adult age group than the older age group (Table 4). This is actually found to be statistically significant with a P-value of 0.023.

Isolation of bacteria from clinical and sub clinical mastitis cases

In the present study, mastitis causing bacteria were isolated from clinical and/or sub clinical mastitis cases. Among the bacterial spices, *Staphylococcus Aureus* 136 (54.4%) dominated followed by *Sterptococus dysgalactia* 62(24.8%), *Staphylococcus intermidius* 21 (8.4%), *Sterptococus uberis* 13 (5.2%) *Staphylococcus epidermides* 11(4.4), *Sterptococus agalactia* 4 (1.6%), *Corynebacterium pyogen* 2 (0.8%) and *E. coli* 1 (0.4%) which was isolated from clinical and sub clinical mastitis cows (Table 5).

DISCUSSION

The study was carried out to determine the prevalence of bovine mastitis and to identify the major bacteria that causes mastitis in Areka woreda and it was revealed that 52.8% animals examined had infections in their udders as evidence of mastitis. This finding closely agrees with those of Handera et al. (2005) and Salih et al. (2011) who reported the prevalence of 52.78% in Ethiopia and 52% in Nigeria, respectively. The current study's prevalence was lower than the finding of Abaineh (1997) who reported 65% in fiche and was higher than the finding of Tolosa et al. (2009), Biffa (1994), Shimel (1990), Darsema (1991) and Fekadu (1995), where they reported 27.3, 29.4, 44.6, 39.8 and 38.65%, respectively.

The present finding showed clinical mastitis cases with the prevalence level of 9.4% in Holstein, local zebu and jersey breeds. The clinical prevalence in this study was similar to that of Tollosa et al. (2009) who reported the prevalence of 9.5% at wolayta sodo and higher than report of Bishi (1998) who reported the prevalence of 5.3% in Addis Ababa (Nesru et al., 1997) who reported 5.3% in central Ethiopia and lower than those reported by Handera et al. (2005) with the prevalence of 16.11% in and around Workineh et al. (2002) reported 25.1% in Addis Ababa.

Sub clinical mastitis was higher as compared to clinical in the three breeds. The prevalence of sub clinical mastitis at cow level based on CMT in the present study (43.5%) was higher than the finding of Demelash et al. (2005) who reported 23% Hundera et al. (2005) who reported 36.6%, Tolloso et al. (2009) who reported the prevalence in wolyta sodo (17.5%).

The variability in the prevalence of bovine mastitis between reports could be attributed to the difference in management of the farm, breeds, season of the study, agro climactic condition or diagnostic test employed. In this study, the prevalence of mastitis as sub-clinical disease entity was higher (43.5%) than clinical forms of mastitis. Robertson (1985) concludes that sub clinical mastitis was usually far higher than clinical mastitis. In Ethiopia, the sub clinical form of mastitis received little attention and efforts have been concentrated on the

Table 2. The prevalence of mastitis based on lactation stage.

Sampled animal	Lactation stage (days)			
	1-120 (Beginning) (%)	120-240 (Mid) (%)	>240 (End) (%)	Total (%)
Infected	78 (37.5)	21 (10.3)	104 (51.2)	203 (52.9)
Non-infected	48 (26.5)	57 (31.4)	76 (41.9)	181 (47.1)
Total	126 (61.9)	78 (26.9)	180 (57.7)	384 (100)

X^2 = 26.642, P<0.05.

Table 3. Showing prevalence of mastitis in different parity group of animals.

Parity group	No. of animals examined		Prevalence (%)
	Affected (%)	Non affected (%)	Total
1 and 2 (G1)	88 (51.6)	84 (48.8)	172 (44.8)
3 and 4 (G2)	91 (58.3)	65 (41.7)	156 (40.6)
5 and 6 (G3)	24 (42.8)	32 (57.1)	56 (14.6)
Total	203 (52.9)	181 (43.1)	384 (100)

X^2 = 3.341, P-value = 0.188.

Table 4. Prevalence of mastitis with respect to different age group.

Age	Positive (%)	Negative (%)	Total (%)
Young	78(38.4)	83(45.8)	161(41.93)
Adult	99 (48.7)	64(35.3)	163(42.45)
Old	26(12.8)	34(18.8)	60(15.6)
Total	203(52.9)	181(47.1)	384(100)

X^2 = 7.496, P = 0.023.

treatment of clinical cases (Hussein et al., 1997) while the economic loss could come from sub clinical mastitis.

Age is a detrimental factor in the distribution of the diseases because at some time it is stress. It was taken into consideration and the prevalence of mastitis was measured for different age groups of lactating cows. The prevalence was found to be much higher in the young (20.3%) and adult (25.8%) age group than the older (6.8%) age group. This is statically significant with P<0.005.

The finding of this study was also assessed for breed predisposition to mastitis but no significant difference in the prevalence was detected between the three breed. It has been reported that mastitis prevalence may be influenced by some inheritable characteristic such as capacity of milk production teat characteristic and udder conformation (Abaineh, 1997). However, the insignificant difference in the prevalence of mastitis between the three breeds reported in this work needs further investigation before a satisfactory explanation is being forwarded. It is worthwhile to mention here that the indigenous zebu stocks are subjected to poor management conditions as compared to Jersey and Holstein cows.

The relationship between the prevalence of mastitis on different lactation stage was studied; the result showed significantly higher infection (p<0.05) in cow with early and late lactation than cow with mid lactation stage. This finding agreed with that of Demelash (1994) where the prevalence of mastitis is higher in the early and late lactation stage. Early stage of lactation and the period of involution of the mammary gland were the most susceptible stage with prevalence of mastitis.

The increase in the prevalence of mastitis with increasing number of lactation reported in this study coincides with result obtained by Osei (1974), Smith et al. (1985) who reported that increase in the prevalence of mastitis accompanied the increase in the lactation number. Sharf et al. (2009) reported that first lactation cows were most resistant to infection. Among the several explanation for the multifarious relationship are increase in teat potency (Murphy, 1994), in this study, the prevalence has been observed to increase up to 4th parity group and decline at subsequent lactation.

With regard to the bacteriological analysis of milk sample, the work revealed that from the CMT positive milk sample the mixed bacterial isolates were the most

Table 5. The relative isolation rate of mastitis causing bacteria.

S/N	Bacterial spices	Frequency	
		No. isolated	Isolation (%)
1	*S. aureus*	136	54.4
2	*S. Dysagalatiae*	62	24.8
3	*S. intermidius*	21	8.4
4	*S. uberis*	13	5.2
5	*S. epidermides*	11	4.4
6	*S. agalactiae*	4	1.6
7	*C. pyogens*	2	0.8
8	*E. coli*	1	0.4
	Overall	250	100

prevalent than each isolated bacteria 114 (29.7%). It was reported that *Streptococcus* species together with *Staphylococcus* species were the most important causes of bovine mastitis (Blood and Radostitis, 1989). And the species of bacteria isolated *S. aureus* was most commonly isolated in clinical and sub clinical case of mastitis in this study case. In this study, *S. aureus* was the predominant pathogen involved constituting 54.4% of all isolate. The high level isolation of *S. aureus* (54.4%) in this study is related with the finding of Ahamed and Mohammed (2007) in Egypt who reported 52.5% and higher than that of Lakew et al. (2009) who reported 39.4%. This finding was not in harmony with reports of Bishi (1998) and Edwards et al. (1982) who found CNS as the predominant species from urban and peri-urban production system in Ethiopia and Bolivia, respectively.

The reason for the higher isolation rate of this organism is the wide ecological distribution inside the mammary gland and skin. In area where hand milking and improper use of drug is practiced to treat the mastitis cases, its domination has been reported by many research scholars. *S.aureus* is adapted to survive in the udder and usually establishes mild sub clinical infection of long duration from which it is shaded through milk serving as sources of infection for other healthy cows and transmitted during the milking process (Radostitis et al., 1994). Hence, the organism has been assuming a position of major importance as a cause of bovine mastitis.

On the other hand, as compared to the proportion of *Staphylococcus,* lower percentage (24.8%) of *Streptococcus* spp. (*Streptococcus dysgalactia*) was isolated. This finding corresponds to the reports made by Geressu (1989), Nesru et al. (1997), Tiruneh (1996) and Hamir et al. (1978) who reported *Streptococcus* spp. as the second major bacteria that causes mastitis and from this species of bacteria, *Streptococcus uberis* and *Streptococcus agalactia* (5.2%) and (1.6%) are next, respectively.

The isolation of *Corynebacterium* spp. was only 1%. The finding was slightly in agree with the findings of Hamir et al. (1978) who reported 1.3%. Coliform spp. with the infection rate in this study was lower as compared to the other bacterial species. *E. coli* (0.5%) out of all bacterial isolated from mastitis positive milk this finding was lower than that of Molalegne et al. (2010), Mengistu (1986) who reported (2.5%) and (3.14%) respactively. In general, the prevalence of mastitis causing agents are high. Thus, the farms should follow the key factors of mastitis program such as good herd management, teat dipping before and after milking, washing milkers hands before and after milking, preparation of clean towel for each lactating cow, milking of infected cow lastly, using dry cow therapy method and treating clinical cases at early stage.

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Full Length Research Paper

Morphological, ultrastructural and molecular variability studies of wild and mutant strains of edible *Pleurotus* species using growth yield, scanning electron microscopy and random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR)

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There is a growing industry of edible mushroom production due to their nutritive value and the recognized fact that mushrooms are natural and healthy foods originating from an environmentally friendly organic farming system. The production of edible mushrooms is threatened by both abiotic and biotic factors, hence the need to improve breeding through genetic tools. In an attempt to determine the morphological and genetic diversity among *Pleurotus* species, fourteen different strains of *Pleurotus ostreatus* and *Pleurotus florida* wild type and their mutants were subjected to different morphological traits, ultrastuctural hyphae network studies and random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) marker. The mycelia growth yield on Petri-plates and in submerged fermentation indicated that the strains PO90 and PF30 were significantly different from the other *Pleurotus* strains used in this study at $P < 0.05$. Also, the microscopy result showed marked differences among the *Pleurotus* strains. The dendrogram based on RAPD analysis generated two different clusters. Out of 4 random primers, distinct polymorphism was observed by primers BG17, BG18, BG 23 and BG25. The percentage similarity among the *Pleurotus* strains varies between 40-100%.

Key words: *Pleurotus* species, submerged fermentation, scanning electron microscopy, biomass yield, random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR).

INTRODUCTION

Mushrooms have been consumed by humans since ancient times, not only as a part of the normal diet but also as a delicacy due to their desirable taste and aroma. Mushrooms have been employed for several useful purposes. They have been employed in pharmaceutical, food and agro allied industries (Alofe et al., 1998;

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Adejoye and Fasidi, 2009; Akinfemi, 2009).

Mushrooms are well known for their medicinal properties and have been widely used in traditional medicine. The medicinal effects of mushrooms include antioxidant, antibacterial, antifungal, antiviral, anti-parasitic, antitumor, immunomodulatory, anti-inflammatory, radical scavenging, anti-diabetic and hepato-protective (Wasser, 2010).

The use of mushrooms with therapeutic properties is growing day by day due to the range of side effects caused by conventional medicines. Many, if not all, higher mushrooms contain biologically active polysaccharides in fruit bodies, cultured mycelia and cultured broth (Wasser 2010a; Smith et al., 2003).

Mushrooms are currently evaluated for their nutritional value and acceptability as well as for their pharmacological properties. In the developing countries of the world such as Nigeria, uncontrolled population growth has created problem of limited food supply, which has led to search for new methods to provide adequate food for humans. The majority of mushroom lovers in developing countries depend solely on mushrooms that grow in the wild (Jonathan and Adeoyo, 2011).

Edible mushrooms are widely eaten by many Nigerian ethnic groups such as the Hausas and Fulanis in the north, and the Yorubas, Ibos, Urhobos, Ijaws and Itsekiris in the south. In Nigeria, mushroom eating is more popular in the villages than in urban areas because, rural people have access to natural vegetation where mushrooms grow.

The genus *Pleurotus* is a heterogeneous group of economic importance. Several species are of nutritional and/or medicinal importance (Cohen et al., 2002; Guzman, 2000). *Pleurotus* species have the ability to absorb microelements from different cultivation media and thus they may present an excellent dietary source (Stajic et al., 2002).

Fungi of the *Pleurotus* genus have an important place among the commercially employed basidiomycetes because they have gastronomic, nutritional and medicinal properties and can be easily cultivated on a large range of substrates (Chang and Hayes, 1978; Kumari and Achal, 2008).

Oyster mushrooms are a more valuable source of protein than either cattle or fish on dry weight basis, and are a good source of almost all the essential amino acids when compared with most vegetables and fruits (Matila et al., 2002).

However, problems in evaluating data published by different investigators working with even the same species of mushroom exist because identification of species is often not accurate. The correct identification of the taxonomic position of mushroom cultures is a task of vital importance.

Before the introduction of molecular tools of identification, there have been a lot of taxonomic chaos; this was because the traditional morphological studies for identification were not well standardized.

An important aspect of the search for natural products with biotechnological potential is that the correct identification of the source species is determined.

The aim of this research was to carry out a detailed study of morphological growth characteristics, vegetative mycelia structures and genomic DNA phylogeny of different strains of *Pleurotus* isolates showing potential biotechnological application, by assessing the growth yield, phase contrast microscopy, scanning electron microscopy and random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR)as an identification tool.

MATERIALS AND METHODS

Microorganisms

The *Pleurotus* species used in this study were obtained from the Mushroom Research Germplasm, CSIR-NEIST, Jorhat, Assam, India. *Pleurotus ostreatus* and *Pleurotus florida* wild-type and their generated mutants were used. The strains were maintained on potato dextrose agar (PDA) slants at 4°C for subsequent uses and sub-cultured bi-monthly.

Mutant strains induction

P. ostreatus and P. florida mutant strains (PO15, PO30, PO45, PO60, PO75, PO90 and PF15, PF30, PF45, PF60, PF75 and PF90) were produced by randomly exposing an actively growing culture (5 days old) of the fungus on PDA plate to an ultraviolet-irradiation (λ = 254 nm) at different time durations of 15 min interval for 90 min (Decla*n* and Alan, 2001). Mycelia plugs obtained from the culture were transferred onto the centre of a fresh PDA plates and incubated at 25°C. After several exposure to UV mutation and sub-culturing of the mutants, mycelia plugs from the survival mutants were selected for further study.

Media

Potato dextrose agar (PDA) Hi-media, India, and formulated culture broth that uses wheat bran wastes were routinely used for experimentation.

Preparation of fermentation medium

Wheat bran filtrate was used for the formulation of fermentation medium. The filtrate was supplemented with the following nutrient compositions: Glucose 50 g/L, Peptone water 2.5g/L, KH_2PO4 2g/L, $MgSO_4.7H_2O$ 1 g/L, $CaCl_2$ 1g/L, Yeast-extract 2.5g/L and (pH adjusted to 5.8). Three agar plugs (6mm diameter), from the *Pleurotus* culture were inoculated into each 200 ml fermentation medium in a 500 ml conical flasks. The medium were now incubated in an orbital shaker at 28°C for 7days at 150 rpm (Talaro and Talaro, 2002).

Harvest of mycelia mat from fermentation medium

After 7 days of fermentation, the medium was harvested by using cheese cloth to sieve in order to obtain the mycelia mat. The dry

weight of the mycelia mat was determined (in mg/l) using digital weighing balance while the percentage medium consumption was calculated as follows:

$$MC\ (\%) = \frac{IMV\text{-}FMV}{IMV} \times \frac{100}{1}$$

Where, % MC = % medium consumption; IMV = initial medium volume; FMV = final medium volume.

Determination of dry mycelia weight

This was carried out by drying the wet mycelia in an oven at 50°C for 24 h to remove the moisture content using pre-weighed filter paper. After drying, the dry-weight was then determined by weighing on weighing balance taking into consideration the weight of the filter paper.

Microscopy studies of wild and mutant strains

Phase contrast microscopy and scanning electron microscopy (SEM) analysis was investigated to study the morphological characteristics in both wild-type and mutants of *P. ostreatus* and *P. florida*.

Sample preparation for phase contrast microscopy

A sterile glass cover slip was inserted on surface of PDA media at an angle of about 30 or 40°C. Fungal culture was inoculated with the help of a needle at the base of the cover slip touching the media. The Petri dish was kept in an incubator at 28°C for 2-3 days for incubation. After sufficient growth of fungal mycelia, the glass cover slip was taken out and placed on glass slide stained with lactophenol-in-cotton blue. It was mounted on a phase-contrast microscope for observation of hyphae and presence of spores. Observation was done first with objective lens (10x) and after with oil immersion lens (100x).

Scanning electron microscopy (SEM)

The SEM is a microscope that uses electrons rather than light to form an image. SEM is a versatile tool for high resolution surface imaging. The SEM uses low energy secondary electrons (SEI) or high energy back scattered electrons (BEI) from the specimen surface for image formation. While SEI image provides information on 50 to 150 3Å thicknesses of the sample, BEI image reveals surface features from larger depth. The advantages of SEM over light microscopy are greater magnification, resolution and much larger depth of field. The study of the ultrastructure of biological material with SEM provides information on fundamental tissue characteristics.

Sample preparation for SEM

For SEM observations, agar plug of the fungus (0.5 cm diameter) were taken for studies and were chemically fixed in solution of 2% glutaraldehyde in 0.2 M sodium cacodylate buffer. They were then washed for 20 min in three changes of sodium cacodylate buffer 0.1 M. Post-fixation was done with 1% osmium tetraoxide in sodium cacodylate buffer at 4°C. It was then washed and dehydrated in a graded alcohol series (varying concentrations of acetone) at 4°C.

Dehydrated samples were dried in a critical point drier at its critical point, that is, 31.5°C at 1100 p.s.i with liquid carbon dioxide, mounted on brass stubs by double sided adhesive tape, and then coated with gold (35 nm thickness) in a sputter coater (JFC-1100) for analyses by SEM. The samples were viewed using scanning electron microscopy (JEOL, JSM 6360).

Molecular diversity study using random amplified polymorphic DNA (RAPD)

Isolation of DNA

The genomic DNA of the two wild-type, that is, *P. ostreatus* and *P. florida* , and their 12 fast growing mutants, making 14 fungal strains all together were extracted using the modified method of Doyle and Doyle (1990). The mycelia from *Pleurotus* spp. were harvested using a scalpel, transferred into sterilized mortar and pestle, and grinded to form a fine paste. DNA extraction buffer consisting of (1 M Tris-Cl pH 8.0; 1 M NaCl; 200 mM EDTA pH 8.0; 10% SDS; 0.1% β-mercaptoethanol) was used.

The paste was transferred into Eppendorf tube, 400 µl DNA extraction buffer was added and centrifuged at 12,000 rpm, at 4°C for 10 min. To the collected supernatant, 300 µl phenol and 300 µl chloroform: isoamylalcohol (24:1) were added and mixed gently. This was centrifuged (12000 rpm, 4°C for 10 min), and the aqueous phase was collected and 500 µl chilled isopropanol was added and incubated at -20°C overnight.

After the incubation, it was centrifuged (12000 rpm, 4°C for 10 min), and the pellet was washed with chilled 70% ethanol and centrifuged for 5 min. The dried pellet was re-suspended in 50 µl of Tris EDTA (10 mM Tris and 1 mM EDTA, pH 8.0) buffer. The isolated DNA was stored at -4°C.

RAPD PCR- Fingerprinting protocol

Random amplified polymorphism protocol uses one primer with an arbitrary sequence unlike normal PCR which uses two. Therefore, amplification in the RAPD occurs anywhere along a genome that contains two complementary sequences to the primer which are within the length limits of PCR. Initial screening was done with 10 primers (Bangalore genei).

Only four primers that gave reproducible and scorable amplifications were further used in the analysis. The reaction recipe for PCR amplification of genomic DNA was as follows: PCR buffer containing $MgCl_2$ - 2.5 µl, 10 mM dNTP mix - 2.5 µl, 10 pM primer - 2.5 µl, distilled water 14.5 µl, Taq polymerase - 1 µl, and finally added was DNA template - 2 µl. Each reaction volume (25 µl) was pippetted into Eppendorf tube and placed in thermal cycler for amplification (Applied Biosystems 9700).

The following thermal profile was applied for RAPD-PCR assay, that is, step 1 - initial denaturation at 94°C for 2 min, Step 2 - Denaturation at 95°C for 30 s, step 3 - annealing at 36°C for 45 s, step 4 - extension at 72°C for 4 - 5 min. After completion of 30 cycles, step 5 - Final extension at 72°C for 8 min.

Electrophoresis of PCR Amplicons

The amplified PCR-Product was analyzed on an agarose gel electrophoresis (1.2% w/v agarose gel). The electrophoresis was run in 1x TBE buffer: 20 mM Tris, 89 mM Boric acid, 20 mM EDTA (pH 8). The gel loading samples consists of 8 µl of the PCR amplified DNA mixed with 1.5 µl 6X gel loading buffer. A 100 bp molecular marker was used. The gel after staining with ethidium bromide was viewed under UV light and documented with a Gel Documentation system (UVP, USA).

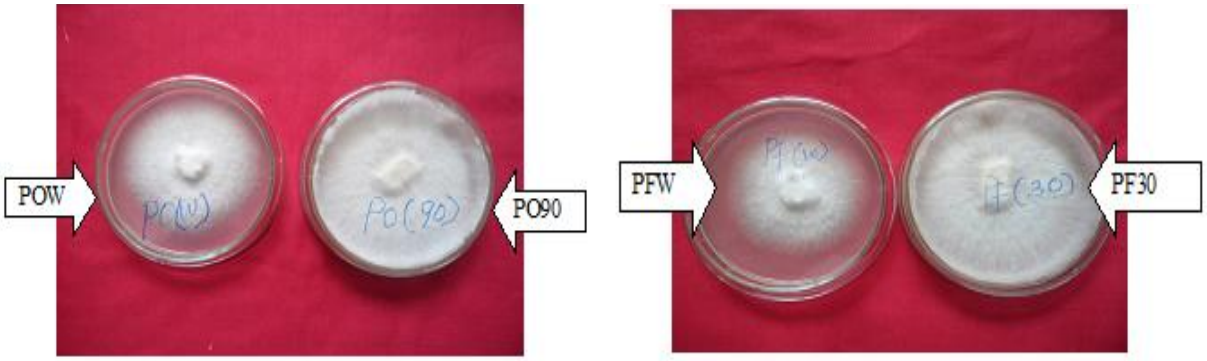

Figure 1. The radial growth rate of wild and mutant strains on potato dextrose agar plate after 5 days of inoculation. (A) *Pleurotus ostreatus* wild and mutant (POW and PO90). (B) *Pleurotus florida* wild and mutant (PFW and PF30).

Table 1. *P. ostreatus* wild type radial growth on plate when compared with the mutants.

Time (h)	Fungal strain						
	POW	PO15	PO30	PO45	PO60	PO75	PO90
24	6.67 ±1.66^{b}	6.66±2.19^{b}	10±1.15^{b}	18.33±2.02^{a}	16±2.91^{b}	15.66±2.96^{b,c,d}	13.33±1.6
48	11.6±1.66^{b,c}	15±1.73^{c}	16.33±1.85^{b,c,d}	15±3.61^{b,c}	25.75±7.55^{b,c,d}	22.33±2.33^{a,d}	18.33±4.93^{b,}
72	20±2.88^{a,c}	26.67±3.71^{a,c}	25±2.30^{a,d}	20±2.88^{a,c}	39.25±11.35^{a,d}	30±4.04^{a,c}	35.±8.66^{a,c}
96	26.66±1.6^{a,c}	29.67±5.04^{a}	33.33±4.41^{a,c}	30±1.15^{a}	47.75±16.12^{a,}	33.33±4.40^{a}	41. 66±3.33^{a,c}
120	30.33±1.45^{a}	31.67±2.03^{a}	31.66±2.84^{a}	31.66±4.33^{a}	55±22.26^{a}	38.333±6.17^{a}	48.33±4.40^{a}

*POW - *Pleurotus ostreatus* wild (POW); *PO15, PO30, PO45, PO60, PO75, PO90 are UV induced mutants respectively. Values shown by different letters in the same columns are significantly different from each other at $P < 0.05$ using Turkey Column Comparison test. Values are given as mean values ± standard deviation.

RAPD data analysis

RAPD bands were scored as present (1) or absent (0). The RAPD banding profile data obtained was entered into the NTSYS-pc version 2.02K package (Rohlf, 1998), a pairwise comparison of isolates was made, and genetic diversity parameters were determined. A dendrogram was constructed by the unweighted pair group method with arithmetic average (UPGMA) (Sneath and Sokal, 1973) to group individuals into discrete clusters.

RESULTS

Selection based on the mycelia radial growth rate of wild and mutant strains of selected *Pleurotus* strains and biomass weight after submerged fermentation

After 5 days of monitoring the growth of wild type and mutants strains for both *P. ostreatus* and *P. florida* on Petri plates at the same time, there was rapid growth in the growth of mutant strains than the wild type as shown in Figure 1. The mycelia radial growth rate was also better in the mutant strains than their wild-type as shown in Tables 1 and 2. Further shown in Figure 2 is the result obtained from the performance of the *Pleurotus* strains in submerged fermentation after harvest, that indicated that mutant strains performance in terms of biomass yield were better, with (PO90) and (PF30) having the highest mycelia mat weight.

The ultrastructural studies of the fungal strains

The morphological differences in the wild and all mutant strains generated were observed by phase contrast microscope under objective lens of 100x and some of the results are shown in Figure 3. It was observed that *Pleurotus* species is a club fungi (basidiomycetes) and septated. Scanning electron microscopy was further done to see the morphological differences between the wild-type and mutant of four selected strains, that is, POW, PO90, PFW and PF30. Figure 4 showed that the hyphae network of the wild-type of *P. ostreatus* is roughened while its mutated strains are smooth. Also shown was that the mycelium strands in wild-type, stand singly, their septa was not obvious, while they have muddled up (compacted) and become bigger in mutated strains. The mutated strains under magnification showed obvious septa when compared with their wild-type. Also in *P.*

Table 2. *P. florida* wild type radial growth on plate when compared with the mutants.

Time (h)	Fungal strains						
	PFW	PF15	PF30	PF45	PF60	PF75	PF90
24	5.67 ±1.15^{b}	4±2.10^{cd}	8.33±4.04^{b}	4.66±1.15^{bc}	3.0±0.33^{bc}	7.33±0.57^{b}	6.66±0.57^{b}
48	6.67 ±1.52^{b}	6.67±2.30^{ad}	12.66±4.61^{bc}	6.66±1.15^{bc}	5±1.0^{b}	12.33±4.04^{bcd}	10±1.0^{b}
72	10±1.0^{bc}	7.33±1.52^{ac}	18.±2.0^{bc}	8.33±4.93^{b}	6.67±2.08^{b}	18.33±7.57^{ad}	11.66.±3.78^{bc}
96	4.33±1.66^{ac}	9±.2.0^{a}	23.33±13.5^{a}	14.33±1.15^{b}	8.33±1.52^{b}	20±5.0 ^{ac}	16.66±1.52^{ac}
120	20.±2.0^{a}	10.66±1.15^{a}	30±5.0^{a}	23.33±5.68^{a}	16.66±2.88^{a}	25±5.0^{a}	20±2.0^{a}

* PFW, *Pleurotus florida* wild (PFW); * PF15, PF30, PF45, PF60, PF75, Pf90 Are UV induced mutants respectively. Values showed by different letters in the same columns are significantly different from each other at P < 0.05 using Turkey Column Comparison test. Values are given as mean values ± standard deviation.

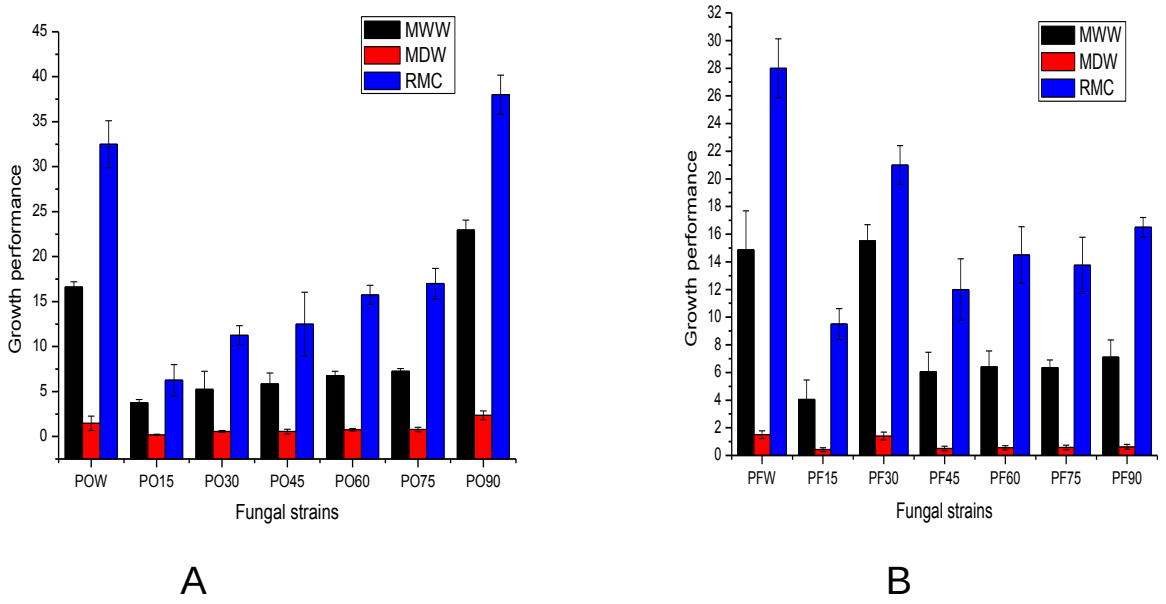

Figure 2. Comparison of mycelia weight and percentage medium consumption of *Pleurotus* fungal strains in submerged fermentation media. (A) *Pleurotus ostreatus* wild and mutant strains. (B) *Pleurotus florida* wild and mutant strains. Error bar represents standard error of means of observed values. *MWW, mycelia wet weight; *RMC, percentage medium consumption; * MDW, mycelia dry weight.

florida, the mycelia of wild-type is rough while its mutant are smooth. In addition, the mutants were well septated while the wild type was not reflected well.

RAPD fingerprinting analysis

The summary of the RAPD-PCR analysis, based on the 4 random primers revealed that Primer BG17 gave 2 polymorphic band with about 40% polymorphism among the *Pleurotus* strains, primer BG 18, 23 and 25 indicated polymorphism of 42.85% and three bands, 28.5% polymorphism and two bands, as well as 36.3% polymorphism and four polymorphic band. From analysis of the four RAPD random primers used in this study (Figure 5), based on the banding profile, also UPGMA based dendrogram was constructed (Figure 6). Also, the summary of primers used and the percentage polymorphism obtained are shown in Table 3.

DISCUSSION

In this study, mutagenesis was used to enhance biomass yield of *P.ostreatus* and *P. florida* mutants using ultraviolet irradiation. The morphological differences in the wild and mutant strains generated were observed by monitoring the mycelia growth rate on the plates, as well as determining the dry mycelia mat weight in mg/l. Also, differences in both wild and mutant strains *of P. ostreatus* and *P. florida* were shown using phase contrast microscope

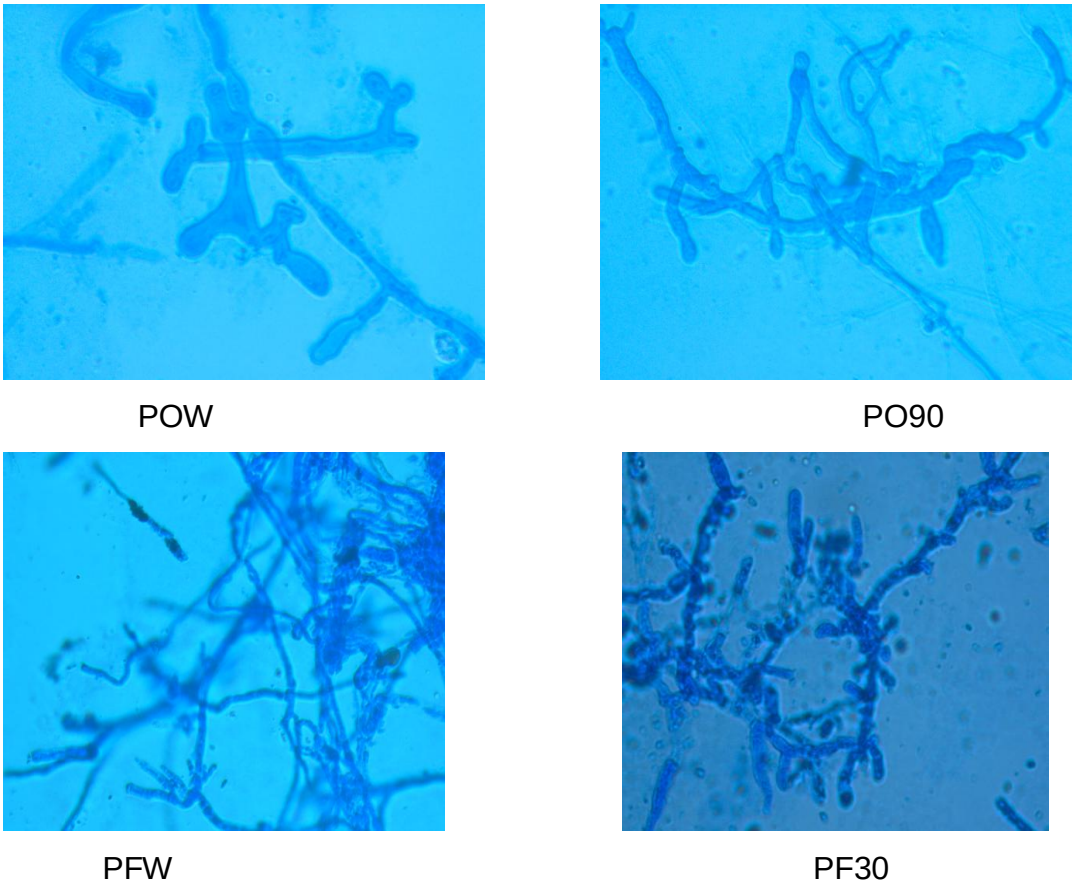

Figure 3. Microscopic observations of wild-type and mutant of *P. florida* and *P.ostreatus* under 100x objective lens of light phase microscope.

microscope and scanning electron microscopy. The result indicated that mutant strains were different from the parental wild strains. The wild strains of both *P. ostreatus* and *P. florida* mycelia were standing singly, less septate and roughened, when compared with their mutated form that were more compacted, septated and much bigger when compared with their wild type. The mycelia mat of PO90 and PF30 were significantly different from the other *Pleurotus* strains used in this study at $p < 0.05$. This result is in agreement with result of Ravishankar et al. (2006) using UV light radiation exposure to improve mycelial biomass yield and higher sporophore production. Also, the result corroborates research by Adebayo et al. (2012) in which UV mutagenesis was used to improve *P. pulmonarius* strains to increase productivity of mycelial and fruiting body yield.

From the molecular diversity studies, the RAPD images obtained from four RAPD random primers based on the presence or absence of bands, a UPGMA based den-drogram was constructed. The constructed dend-rogram consisted of two major clusters. Cluster 1 com-prised of POW, PO15, PO60, PO75, PO45, PO90, PO30 and cluster II comprised of PFW, PF30, PF45, PF75, PF15, PF60 and PF 90. The dendrogram indicated that POW, PO15, PO60 and PO75 were 100% similar. Other *Pleurotus* strains in this study ranges between 40-80% in similarity.

This indicated that truly, there was diversity in the different strains of *Pleurotus* species. The summary of the RAPD-PCR analysis, revealed that primer 17 gave 2 polymorphic band with about 40% polymorphism among the *Pleurotus* strains, primer 18, 23 and 25 indicated polymorphism of 42.85% and 3 bands, 28.5% polymorphism and 2 bands, as well as 36.3% poly-morphism as well as 4 polymorphic band. A similar result was reported by Chandra et al. (2010) using RAPD markers to discriminate eight *Pleurotus* species and also found variations in their banding patterns.

The genetic diversity result obtained in this study corroborates earlier studies reported by Stajic et al. (2005), Khan et al. (2011) and Hyeon-Su et al. (2007) using RAPD molecular marker in the diversity study of mushrooms. Stajic et al. (2005) working with 37 strains of 10 *Pleurotus* species obtained a dendrogram that grouped the strains into 6 different genetic clusters. He reported that morphological based grouping did not

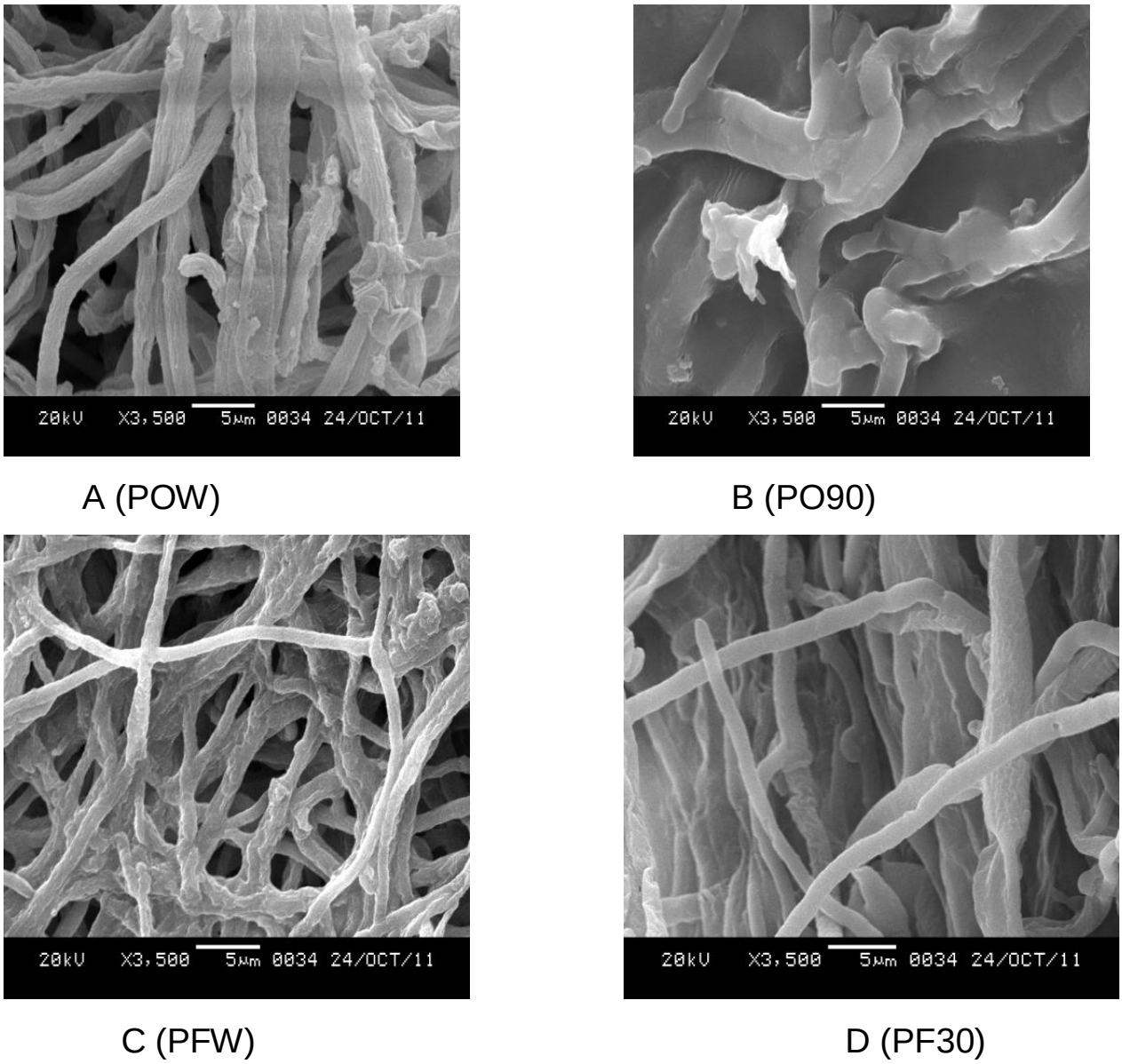

Figure 4. Scanning electron microscopy of hyphae network of wild and mutant strains of *Pleurotus ostreatus* and *Pleurotus florida*.

match genomic relationship among the species. Also, working with RAPD-PCR, Hyeon-Su et al. (2007) demonstrated that analysis of DNA amplifying pattern could be classified into five different clusters based on genetic similarity analysis and this indicated genetic diversity.

The research of Khan et al. (2011) using RAPD-PCR to analyzed genomic DNA of seven species; found out that the number of bands and banding patterns were variable depending upon the primer and type of species. The similarity matrix differentiated the species into three distinct clusters.

Studies confirmed that this techniques is a good genotypic identification analysis that is better than morphological and physiological identification, that are influenced by cultivation conditions (Iqbal et al., 2010)

Conclusion

This study confirmed that RAPD-PCR, that is a simple, cost effective technique is a good genotypic identification analysis that is better than morphological and physiological identification, that are influenced by cultivation conditions.

Mushroom growers can use RAPD analysis and morphological evaluation to characterize mushrooms in order to maintain good quality mushroom breed.

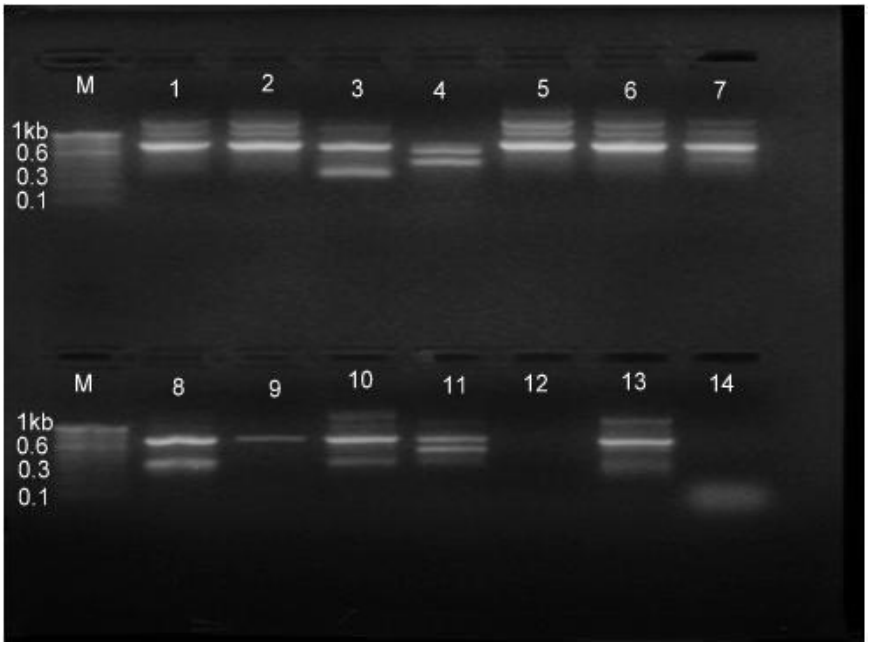

Figure 5. RAPD-PCR banding profile obtained with primer number BG17 of DNA isolated from *P. ostreatus* and *P. florida* wild and mutant strains. Lane M: 100 bp ladder, Lane 1: PfW, Lane 2: Pf15, Lane 3: Pf30, Lane 4: Pf45, Lane 5: Pf60, Lane 6: Pf75, Lane 7: Pf90, Lane 8: POW, Lane 9: PO15, Lane 10: PO30, Lane 11: PO45, Lane12: PO60, Lane 13: PO75, Lane 14: PO90.

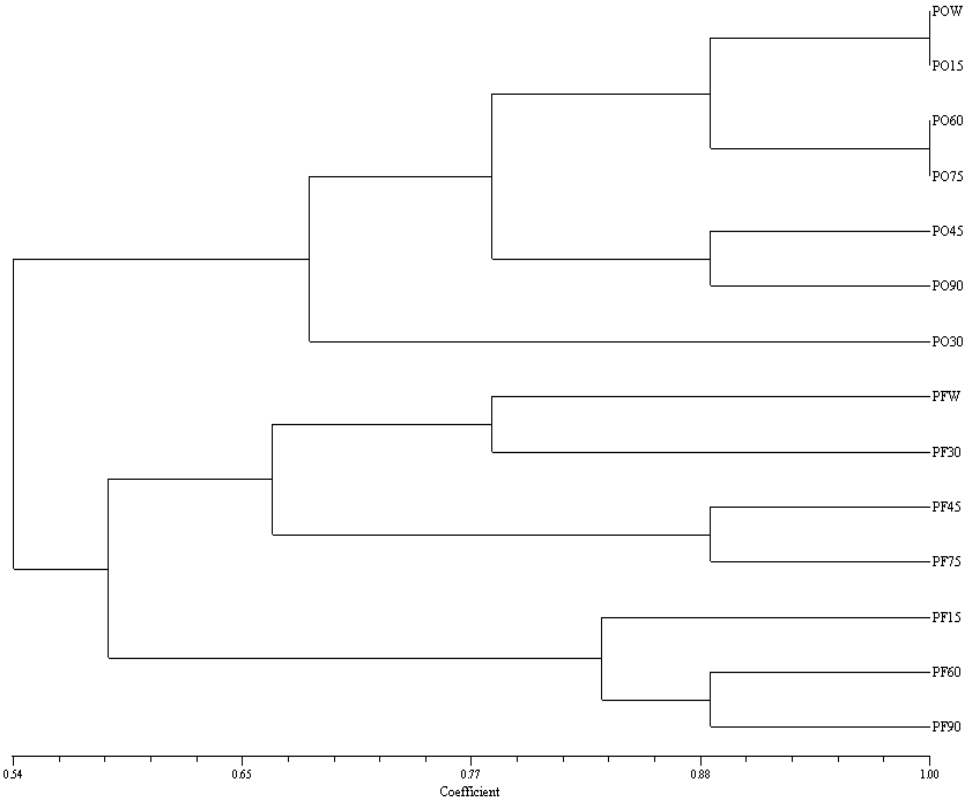

Figure 6. Dendrogram of wild and mutant strains of *Pleurotus* species constructed by combining banding profile of the four primers using neighbour-joining cluster analysis method produced from Jackard estimates using NTSYS Spc Version 2.20.

Table 3. Summary of polymorphism result of the four primers used.

Primer code	Polymorphic bands	Total bands	Percentage Polymorphism (%)	Range of band size
BG 17	2	5	40	300-1700
BG 18	3	7	42.85	300-2500
BG 23	2	7	28.5	300-2200
BG 25	4	11	36.3	500-1800
Total	11	30	147.65	
Mean/primer		7.5		
Average polymorphism			36.9	

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Short Communication

The Effect of 50 Hz electromagnetic field on *Helicobacter pylori*

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We aimed to determine the impact of 50 Hz, 3 mT electromagnetic field (EMF) on the growth of *Helicobacter pylori* and the changes in morphology, survival and viability after exposure in both 20 and 120 min of *in vitro* experiments. The bacterial suspension (~10^8CFU/mL) of cultured *H. pylori* NCTC 11637 in Brucella Broth were exposed to 50 Hz EMF for 20 and 120 min in a microaerophilic condition at 25-28°C. Viability or culturability of 10^4 and 10^5 dilutions was determined after 20 and 120 min by spread plate colony count method. The exposure to 50 Hz EMF for 120 min had inhibitory effect on *H. pylori* colonies with reduction in bacterial viability as well as morphologic changes which were observed from typical spiral form to elongated bacillary form.

Key words: *Helicobacter pylori*, electromagnetic fields.

INTRODUCTION

Biologic system response to the exposure of electromagnetic field (EMF) is changed by the frequency and intensity of EMF, exposure time and the structure of biological system (Nakasono and Saiki, 2000; Yi-Li et al., 1997). Magnetic fields are in use for magnet resonance tomography, magnetic stimulation of brain areas and magnetic drug targeting in medicine. There are several studies that prove the therapeutic benefit of magnetic fields (Obermeier et al., 2009).

There are many biological studies to clarify the biological effects of magnetic fields. The exposure effects are not understood (Nakasono and Saiki, 2000). There are EMF in high voltage line and electricity machine such as television at home and office for human use (Cellini et al., 2008; Erol et al., 2003). The biological effect of magnetic field is found to be general stress factor in bacteria, yeast and HL60 human leukemia cells. The general stress response via temperature, toxins, heavy metals, free radicals is found in all bacteria, plant and animal cells. The mechanism of the stress response is well known in *Escherichia coli* (Nakasono and Saiki, 2000). *E. coli* ATCC 700926 was shown to respond to exposure of 50 Hz and 0.1-1.0 mT modifying morphology from bacillary to coccoid as an immediate effect (Cellini et al., 2008).

Helicobacter pylori infection is very common worldwide, occurring in 25-50% of the population in developed countries, 80-90% in developing regions (Bardhan, 1997). Its natural habitat is human stomach, but it can survive in dental plaque, feces in human and animal and water (Adams et al., 2003; Kabir, 2003). There is a strong link between *H. pylori* and gastric cancer in many countries. By contrast, low gastric cancer rates have been reported in some countries with a high prevalence of *H. pylori* infection, such as India and Bangladesh. (Machida-Montani et al.,2004). Chronic *H. pylori* induced inflammation can eventually lead to atrophic gastritis and intestinal metaplasia which occurs in approximately half of the *H. pylori*-colonized population. *H. pylori* increases the risk of gastric cancer development via atrophy and intestinal metaplasia in *H. pylori* positive person than uninfected persons. Gastric cancer is the second most

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Figure 1. The cylindrical solenoid electromagnetic field aparatus used for exposure of *H. pylori* NCTC 11637 to EMF (50 Hz of frequency and 3.0 mT of intensity). The bacterial suspensions of *H. pylori* contained in anaerobic jar and Cook roasting bag transparent to the EMF was located on the middle part of the magnetic field inside the coil system.

common cause of cancer-related deaths worldwide (Peek and Crabtree, 2006). This difference in gastric cancer rates in populations with similar high prevalences of *H. pylori* infection could be related to the difference in the diversity of *H. pylori* strains, ethnicity and environmental factors (Machida-Montani et al.,2004) Consequently, the viability of *H. pylori* in different environment is related to its genetic variability and its environment adaptability. *H. pylori* modifies itself from spiral to coccoid form in which is viable but not culturable (VBNC) state. This cellular response to environmental stress is emphasized when bacterial cells organize themselves into microbial communities forming biofilm. Therefore, the capability for this pathogen to produce a polymeric matrix forming biofilm may play a very important role in its survival in the environment. The influence of electromagnetic microwaves on living organisms have been widely described resulting with different effects. In particular, for prokaryotic systems, the exposure to electromagnetic fields produces stress effects causing phenotypic and transcriptional changes on free cells and affecting the surface adhesion on cells organized in biofilm (Di Campli et al., 2010).

We aimed to detect the role of exposure of 50 Hz 3.0 mT electromagnetic fields (EMF) stress on the growth of *H. pylori* and its adaptibility to environmental changes in the morphology, viability and proliferation of *H. pylori*.

MAGNETIC FIELD EXPOSURE SYSTEM AND FIELD CHARACTERISTICS

The magnetic fields were generated with 50 Hz alternative flow frequency to 30-80 Gauss (G) EMF by a pair of Helmholtz cylindrical solenoid system which was used in the Department of Anatomy, Faculty of Medicine, for experimental design. Electromagnetic field system was composed of 95 cm scaled in diameter with two copper solenoids which is positioned 50 cm distance between the coils (Figure 1). Solenoids were connected in series to a generator delivering an AC current. The output current was 6.43 A at 50 Hz. The magnetic field intensity and the stress of electromagnetic field were measured by transformator and a digital teslameter (FW Bell, 5170, Pacific Scientific-OECO, Milwaukie, OR, USA). The teslameter accuracy was ±2% for AC (Alihemmati, 2009; Kiray et al., 2013; Tayefi et al., 2010).

Bacterial strain and experimental procedures

H. pylori NCTC 11637 standard strain was cultured on Columbia Blood Agar including *H. pylori* Selective Supplement (DENT, Oxoid) and 7% defibrinated horse blood (Horse Blood Defibrinated, Oxoid); incubated in anaerobic jar (2.5 L, Oxoid) and roasting bag (25 x 38 cm, Cook) separately for four days at 37°C in micro-aerophilic conditions with GasPak Campy Container System (Becton Dickinson and Company). Bacterial suspension was prepared and adjusted in 30 ml Brucella Broth (Becton Dickinson and Company,USA) from cultured *H. pylori* colonies to obtain McFarland 2 (~10^8 CFU/mL) by Densimat (Biomerieux, France). *H. pylori* suspension was distributed into each of six screw capped sterile test tubes.

In microaerophilic condition, bacterial suspension one in Cook roasting bag and one in anaerobic jar were placed and exposed to 50 Hz 3.0 mT EMF for 20 min (acute exposure) and 120 min at room temperature (25-28°C). The controls were carried out in anaerobic jar at the beginning of the experiments at 20 and 120 min without exposure to EMF outside the Helmholtz cylindrical solenoid system at 25°C in the same room (Figure 1). The experimental design was carried out and each experiment was performed in dublicate.

Culturable, total count and microscobic observations

For the culturable cell count, at each time interval, CFU/mL was determined by plating 200 µl of 10-fold concentrated Brucella Broth cultures on four plates of serial dilutions of each *H. pylori* suspensions cultured on Columbia Blood Agar and incubated at 37°C for 3 days.

After 20 and 120 min of exposure, each *H. pylori* suspensions incubated in anaerobic jar, roasting bag and control were separately diluted for colony counting. 4th (10^4) and 5th (10^5) dilutions of each *H. pylori* suspensions were inoculated as duplicate on 7% horse blood Columbia Blood Agar and incubated at 37°C for 3 days in microaerophilic conditions with GasPak Campy Container System. The *H. pylori* colonies that appeared on the cultured plates were counted and expressed in colony-forming units (CFU). In addition, one slide was prepared for wet mount examination for motility, one slide was air dried and stained with modified Gram staining to examine morphological changes for initial time, exposure duration in 20 and 120 min. Bacterial shape was evaluated with light microscope at each time point. The slides for each culture were examined and ten fields for each slides were

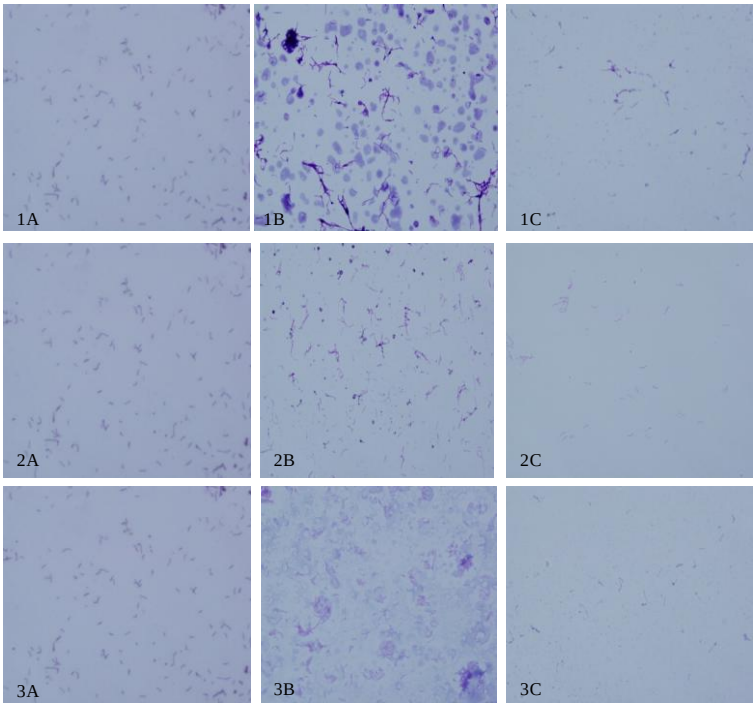

Figure 2. The view of the McFarland 2 suspension of NCTC 11637 *H. pylori* standard strain morphology which was incubated in (1) anaerobic jar (x100) with exposure to EMF (2) Cook roasting bag (x100) with exposure to EMF (3) anaerobic jar without exposure to EMF: (A) initial time, (B) 20 min and (C) 120 min.

counted blindly by four of us. The number of bacillary and elongated forms were determined.

Viability testing

H. pylori in Brucella Broth exposed and non-exposed to EMF were examined for their viability with 2-(P-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) (0.8 mg/ml) stain. INT was added to each *H. pylori* bacterial suspension tubes which were exposed to EMF in 20 and 120 min both in Cook roasting bag, anaerobic jar and control tubes and incubated for 2 h in microaerophilic condition at 37°C. Each were inoculated as duplicate on 7% horse blood Columbia Blood Agar and incubated at 37°C for three days in microaerophilic conditions with GasPak Campy Container System. For each determination colonies were counted if present for visible growth or not.

RESULTS AND DISCUSSION

The stimulation effects of alternating electric current on microbial growth have been shown in some studies. The growth of different bacteria is stimulated by 50 Hz, the enzymatic activity of living cells is increased by alternating current and the lag phase of *E. coli* depending on inoculum size, shaking rate during cultivation and composition of the medium (Loghavi et al., 2007). However, in our study, there was no stimulation effects on the growth of *H. pylori* and also *H. pylori* did not change from bacillary form to coccoid form due to the exposure to EMF. Although the colonies of *H. pylori* in Cook roasting bags were smaller than the size of colonies of *H. pylori* in anaerobic jar, typical spiral morphology was observed in both anaerobic jar and Cook roasting bags. We evaluated the slides of *H. pylori* cultered colonies from the anaerobic jar and Cook roasting bag and also the slides of *H. pylori* bacterial suspensions at initial time, at durations of 20 and 120 min of electromagnetic exposures stained with modified Gram stain, respectively (Figure 2). However, we interestingly observed that the morphology of bacteria was changed from typical spiral morphology to elongated shape morphology in both anaerobic jar and roasting bag for only 120 min.

It was reported that *Lactobacillus acidophilus* lag phase and spesific growth rate were not affected significantly by exposure to 60 Hz (Loghavi et al., 2007). It was reported that *Staphylococcus aureus* concentrations in broth medium could be reduced under the influence of the four different applied fields within 24 h of experiment. The strongest effects were observed for the direct current electric field which could decrease CFU/mL of 37%, and

the low-frequency EMF with additional induced electric alternating field with a decrease of Staphylococci concentration by 36% (Obermeier et al., 2009). We observed that the exposure to 50 Hz -3 mT EMF resulted in morphological changes in both anaerobic jar and Cook roasting bag cultured *H. pylori* strains and a decrease in growth (10^3 CFU/mL in 4th dilution, 10^2 CFU/mL in 5th dilution) was observed in anaerobic jar at 120 min. The number of *H. pylori* colonies which were exposed to 50 Hz EMF in 20 min in both anaerobic jar and roasting bag were not decreased according to the control group. However, the number of *H. pylori* colonies which were exposed to 50 Hz EMF in 120 min in anaerobic jar were decreased according to the control group and Cook roasting bag. No difference was found between the control groups for 20 and 120 min. After INT staining for 2 h, we observed that bacteria were seen in both pellet and cloudy supernatant in all tubes from Cook roasting bag, anaerobic jar and control tubes.

We concluded that exposure to 50 Hz EMF in 20 and 120 min were related to minumum lethal activity of *H. pylori* NCTC 11637 standard strain although the morphology of bacteria was changed due to exposure to 50 Hz EMF in 120 min. We also suggested that the Cook roasting bag was more easily acquired, cheap, disposible and useful than anaerobic jar and only the incubation period of culture might be prolonged one more day for the appearence and growth of *H. pylori* colonies.

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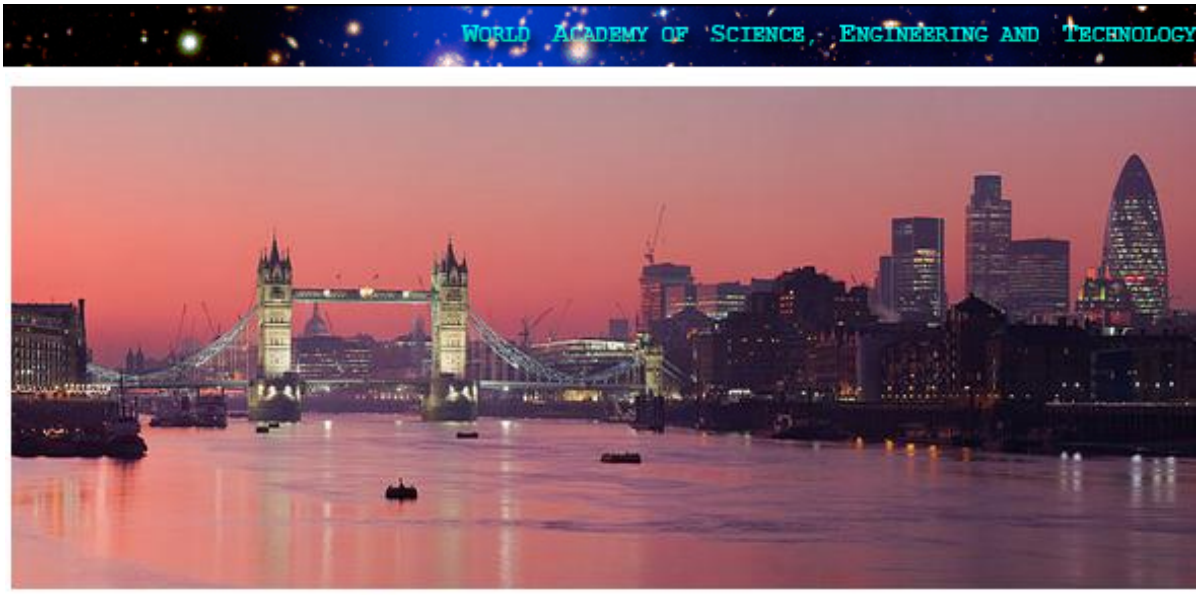

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