African Journal of Microbiology Research

Volume 8 Number 15, 9 April, 2014

ISSN 1996-0808

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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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academicJournals

Vol. 8(15), pp. 1544-1561, 9 April, 2014
DOI: 10.5897/AJMR2013.6573
Article Number: FB69E5243798
ISSN 1996-0808
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African Journal of Microbiology Research

Review

Fluorescent pseudomonads: Milestones achieved in the last two decades

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Received 20 December 2013; Accepted 17 March, 2014

Environmental concerns have led to the need of sustainable use of natural resources. The conventional agriculture practice caused considerable pollution and unavoidable impacts on soil, water, animal as well as human health. Fluorescent pseudomonads, a group of root-associated bacteria that can colonize the roots of crop plants and produce antifungal metabolites represent a real alternative to the application of chemical fungicides. Fluorescent pseudomonads have been studied for decades for their plant growth-promoting effects through effective suppression of soil borne plant diseases. During root colonization, these bacteria produce antifungal antibiotics that can indirectly suppress fungal pathogens by scavenging iron in the rhizosphere environment through the release of siderophores, elicit induced systemic resistance in the host plant or interfere specifically with fungal pathogenicity factors. Before engaging in these activities, the bacteria go through several regulatory processes at the transcriptional and post-transcriptional levels. Current genomic analyses of rhizosphere competence and biocontrol traits of fluorescent pseudomonads will likely lead to the development of novel tools for effective management of deleterious phytopathogens and a better exploitation of their plant-beneficial properties for sustainable agriculture. This review addresses the main findings on fluorescent pseudomonads for the last two decades. It summarizes and discusses significant aspects of this general topic, including taxonomic status, genetic diversity of fluorescent pseudomonads in India, different mechanisms of biological control and commercial use of fluorescent pseudomonads in agriculture.

Key words: Fluorescent pseudomonads, biological control agent, secondary metabolites, induction of systemic resistance, post-transcriptional regulation.

INTRODUCTION

This topic specifically will focus on fluorescent pseudomonads because they are present in many environments, especially in the plant rhizosphere. Many studies involve these bacteria that are able to improve plant growth and plant health and are implicated in the natural supressiveness of certain soils to many soil-borne

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diseases whereas others participate in the biodegradation of natural and man-made toxic chemical compounds. The γ-subclass of the Proteobacteria includes fluorescent pseudomonads (FLPs) along with several non-fluorescent species. The fluorescent *Pseudomonas* group includes: (1) phytopathogenic cytochrome *c* oxidase-positive species, viz. *Pseudomonas cichorii*, *Pseudomonas marginalis* and *Pseudomonas tolaasii*, (2) non-phytopathogenic, nonnecrogenic strains, viz. *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas chlororaphis*, *Pseudomonas aureofaciens* and *P. Pseudomonas aeruginosa* type species and (3) phytopathogenic necrogenic fluorescent *Pseudomonas* spp. without cytochrome *c* oxidase, viz. *Pseudomonas syringae* and *Pseudomonas viridiflava*. The non-fluorescent *Pseudomonas* group includes *Pseudomonas stutzeri*, *Pseudomonas mendocina*, *Pseudomonas alcaligenes* and *Pseudomonas seudoalcaligenes* (Palleroni, 1993). Phenotypic characterization has been employed to cluster and identify bacteria according to several features, viz. morphology, pigmentation and reaction to dyes and nutritional requirements. It was observed that *P. fluorescens* and *P. putida* are very heterogeneous. *P. putida* was subdivided into biotypes A and B, which became biovars A and B. *P. fluorescens* was also subdivided into 7 biotypes. The biotypes A, B, C, D and F were then called biovars (bv. I to V) and biotypes D and E became the *P. chlororophis* and *P. aureofaciens*, respectively which were then clustered as the *P. chlororaphis* (Jonhson and Palleroni, 1989). *P. fluorescens* bv. V includes strains that often cannot be classified because properties that are essential for the differentiation from other biovars are not identified. This system of multiple biovars reveals a high phenotypic heterogeneity, and probably reflects high genomic diversity. Phenotypic studies revealed this high variability with these two species, which resulted in subdivision in these biovars and sub-grouped of each one (Grimont et al., 1996; Janse et al., 1992; Latour et al., 1996; Lemanceau et al., 1995; Sorensen et al., 1991). To elucidate the real taxonomic condition of these subgroups it is important to characterize genotypically the species and biovars. DNA-rRNA hybridization studies led to the delineation of the genus *Pseudomonas* into five homology groups (Palleroni et al*.,* 1973). The genus *Pseudomonas sensu stricto* corresponds to the Palleroni *sensu* rRNA homology group I and contains all the fluorescent species (Palleroni, 1993). Different studies show very high genomic variability within biovars of *P. fluorescens* and *P. putida* and probably some biovars correspond to undescribed species (Champion et al*.,* 1980; Palleroni et al*.,* 1972). Bossis et al*.* (2000) also got similar results. The level of similarity of rhizosphere isolates identified as *P. fluorescens* or *P. putida,* by the phenotypic criteria of Palleroni was never greater than 55% as compared to the type strains of *P. fluorescens* and *P. putida* (Bossis et al*.,* 2000). Future studies on the ecology of fluorescent pseudomonads require reliable, fast and cheap methods to identify large numbers of isolates. The description of new species within the distinct species *P. fluorescens* and *P. putida* remains to be done in order to elucidate the taxonomic status of this group.

The current state of the pseudomonas taxonomy makes it difficult to assess the phylogenetic distribution of biocontrol agents within *P. fluorescens* and closely-related fluorescent pseudomonads (Bossis et al., 2000). However, it seems clear that these taxa include both biocontrol agents and strains without any obvious biocontrol potential, regardless of whether only true *P. fluorescens* or also related fluorescent pseudomonads are considered (Sanguin et al*.,* 2008). It is important to note that *P. fluorescens* and neighbouring species are thought to include also strains with human pathogenicity potential (Wei et al., 2002; Bodilis et al., 2004), but the evidence to date is not fully convincing in the current taxonomic context and this issue deserves further clarification. *P. fluorescens* and closely-related fluorescent pseudomonads appear to be predominantly clonal (Frapolli et al., 2007). Yet, horizontal gene transfer may take place and such a possibility has been raised for genes involved in the interaction with the plant and/or phytopathogens (Ramette et al., 2003; Blaha et al., 2006). This includes also the hypothesis that genes involved in the synthesis of biocontrol compounds might have been acquired from the plant itself (Cook et al., 1995; Ramette et al., 2001).

GENETIC CHARACTERIZATION OF FLUORESCENT PSEUDOMONADS

The nucleotide sequences of various genes, especially those of small-subunit (SSU) rRNA have been widely used to identify and classify microorganisms (Woese, 1987). The study of SSU rRNA sequences resulted in many findings, such as the *Archaea*, a group of prokaryotes that is separated from *Bacteria*. This gene is most widely used. Its sequence is a mosaic of highly conserved regions interspersed with variable and hypervariable stretches that makes it convenient for PCR primer design (Gürtler and Stanisich, 1996). The 16S rRNA gene from *Pseudomonas* spp. contains 1492 nucleotide positions, of which 148 are variable and 65 positions of these are within three hypervariable regions (Moore et al*.,* 1996). The "*Pseudomonas* hypervariable (hv) regions" were defined as: hv 1, *Escherichia coli* 16S rRNA gene sequence positions 71- 95; hv 2, *E. coli* 16S rRNA gene sequence positions 455 - 475; and hv 3. *E.*

coli 16S rRNA gene sequence positions 998-1043 (Godfrey and Marshall, 2002; Moore et al., 1996). These positions are located, respectively, within the regions V1: helix 6, V3: helix 18 and V6: helices P35-1 and P35-2 (Godfrey and Marshall, 2002; Neefs et al., 1990). The regions hv1 is considered one of the most variable sequences in 16S rRNAs of bacteria across the phylogenetic spectrum (Gutell et al*.,* 1985; Woese, 1987) and is useful for differing intrageneric lineages and discerning the type strains of some species of *Pseudomonas* (Moore et al., 1996). Godfrey and Marshall (2002) used primers based in these regions to study the diversity of pseudomonads isolates from different parts of the world. The large database of 16S rRNA sequences is important for the analysis of environmental isolates and recognition of new sources of diversity (Moore et al*.,* 1996). Nevertheless, there are some pitfalls in the use of rDNA for studies of biodiversity (García-Martínez et al*.,* 1999). The gene size is constant and consequently different genes could not be easily separated by size. In addition, the 16S genes have hypervariable and extremely informative regions. They are often not divergent enough to separate in close relationships (García-Martínez et al., 1999; Normand et al., 1996). Other techniques are utilized to solve these limitations for characterizing isolates from the environment. The region located between the 16S and 23S (ITS1) rRNA genes is very variable in size and sequence even within closely related taxonomic groups (Gürtler and Stanisich, 1996). Size pattern can characterize different communities of *Eubacteria* or *Archaea* and the widely divergent sequence allows the detection of species-like units very precisely by PCR, oligo-probes or long DNA probe hybridization (García-Martínez et al*.,* 1999; Jensen et al*.,* 1993). Locatelli et al. (2002) designed a set of primers specific for *Pseudomonas* that allowed the amplification of ITS1 together with a significant part of 16S rDNA. The primers were conserved for all the *Pseudomonas* sequences tested and they showed specificity and efficiency for the amplification of 1100 to 1300 bp fragment to the *Pseudomonas* spp. Interspersed repetitive DNA sequence elements are present in prokaryote genomes and can be used as primers sites for genomic DNA amplification (Versalovic et al., 1991). Three families of repetitive sequences have been studied in most detail, including the 35 - 40 bp repetitive extragenic palindromic (REP) sequence, the 124-127 bp, enterobacterial repetitive intergenic consensus (ERIC) sequence and the 154 bp BOX element comprised of three subunits (boxA, boxB and boxC) (Lupski and Weinstock, 1992). These sequences are located in distinct, intergenic positions all around the chromosome. Louws et al. (1994) demonstrated that REP, ERIC and BOX-PCR, referred to as rep-PCR collectively, were useful for the rapid molecular characterization of plant pathogenic bacteria, like *Xanthomonas* and *Pseudomonas*, especially at the pathovar level.

Many other approaches are used to analyze the diversity of the pseudomonads. Delorme et al. (2003) studied the membrane-bound nitrate reductase (*narG*) and nitrous oxide reductase (*nosZ*) genes diversity, implicated in the denitrification process. They compared the similarity indexes of the *narG* and *nosZ* genes in different strains and the genes led to the identification of two different groups of strains. The first group presented similarity between the genes suggesting similar evolutionary pathway. The second group, on the other hand showed higher diversity of the *nosZ* gene as compared to the *narG* gene, suggesting different evolutionary rates.

REPORTS OF GENETIC DIVERSITY OF FLUORESCENT PSEUDOMONADS FROM INDIA

The rhizosphere community structure of wheat and the influence of genotype on community structure have been studied extensively for the Indo-Gangetic region (Mittal and Johri, 2008). It was observed that the wheat genotype did not appreciably influence the total and pseudomonad populations. Population structure was only marginally different in the rhizosphere (RS) and rhizoplane (RP) fractions, which could be explained on the basis of a wheat genotype-dependent influence. Analysis of cultivable genetic diversity by employing ARDRA and rep- PCR showed that for any single variety, the distribution of various bacterial morphotypes was fairly even, although the RP fraction was generally more diverse than the RS fraction. Diversity indices showed var. UP2338 to be the richest (E), whereas var. HD2627 was most diverse (H′). Numerical analysis of phenotypic characters revealed that most of the isolates exhibiting greater similarity with *Pseudomonas* reference strains belonged to var. UP2338; this was later confirmed by 16S rDNA sequencing. Sequencing data also revealed that among γ -proteobacteria, pseudomonads were most prominent along with *Pseudoxanthomonas* and *Stenotrophomonas* (Mittal and Johri, 2008). In another study, the genetic diversity of plant growth promoting rhizobaterial fluorescent pseudomonads of sugarcane cultivated in Vagaikulam around Madurai, India has also been extensively studied. They showed using the 16s rDNA sequence similarity of the isolates that, they belonged to *Pseudomonas plecoglossicida, P. fluorescens, P. libaniensis* and *P. aeruginosa*. Further differentiation of the isolates was done through different genomic DNA finger printing techniques and it was observed that *Pseudomonas plecoglossicida* is a dominant species (Neelamegam et al*.,* 2012). Recently, *rpoB*-RFLP is becoming an emerging tool for determining

diversity indices of fluorescent pseudomonads. In a recent study with 543 isolates, collected from different parts of northern and eastern Indo-Gangetic plains showed that 26 different clusters were formed from 16S rDNA-RFLP whereas 27 clusters were generated by the *rpoB*-RFLP with similarity percent ranging from 3 to 100%. 16S rDNA sequencing showed 9 different species of *Pseudomonas,* whereas, *rpoB* sequencing showed 13 different species of *Pseudomonas*. Phylogenetic analysis based on 16S rDNA gene sequences generated 15 branches showing more than 70% of boot strap value, whereas 18 branches in the *rpoB* based phylogenetic tree were supported by bootstrap values above 70%. Diversity indices based on *rpoB* were higher than the ribosomal RNA gene (Yadav et al*.,* 2013).

INTERACTION BETWEEN PLANT AND FLUORESCENT PSEUDOMONADS: THE SELECTION MACHINERY OF HOST PLANT

Mechanism of recognition

Many plant-associated *Pseudomonas* promote plant growth by suppressing pathogenic microorganisms, synthesizing growth-stimulating plant hormones and promoting increased plant disease resistance. Others inhibit plant growth and cause disease symptoms ranging from rot and necrosis to developmental dystrophies such as galls. It is not easy to draw for the plant system a clear distinction between pathogenic and plant growth-promoting *Pseudomonas* (PGPP) as they colonize the same ecological niches and possess similar mechanisms for plant colonization. Over all, the net cost or benefit of interactions with PGPP is affected by the nutritional status of the soil, toxic effects of the bacterium and presence of fungal pathogens, further complicated by plant age, environmental factors, induced stress resistance and cross-talk between plant signal transduction pathways (Preston, 2004).

Recently, green fluorescent protein (GFP) and bioluminescence techniques have been employed effectively to investigate these issues. GFP technology, together with confocal laser scanning microscopy (CLSM), has facilitated the detection of the mechanism of recognition and colonization up to single cell level (Bloemberg et al*.,* 2000; Normander et al*.,* 1999).

Recognition machinery: Fllagelin and LPS

Plants have evolved the capacity to recognize and respond to a wide range of generic microbial molecules, the so-called pathogen-associated molecular patterns (PAMPs). Two of the most widely studied PAMPs produced by *Pseudomonas* are flagellins, subunits of the polar flagella produced by motile *Pseudomonas* and lipopolysaccharides (LPSs), constituents of the bacterial envelope.

Flagellin recognition in plants is mediated by flagellin-sensitive2 (FLS2), a membrane-associated kinase with an extracellular leucine-rich repeat (LRR) domain. FLS2 is a member of the Toll family of receptor kinases, which have been linked to developmental signaling and pathogen recognition in plants. Flagellin recognition by plants is host and strain-specific, e.g. the Ws-0 ecotype of *Arabidopsis* is insensitive to *Pseudomonas* flagellins, showing that flagellin recognition is not a universal characteristic of plants, even within a plant species (Gómez-Gómez et al*.,* 1999). Flagella are important for initial colonization of roots and leaf surfaces, but not for endophytic multiplication. Regulation of flagella expression could be an additional mechanism used to evade plant recognition of *Pseudomonas*.

A second commonly recognized factor is LPS. LPS recognition has mostly been studied in the context of plant pathogens, where it has been shown to induce plant synthesis of anti-microbial factors and to suppress the development of programmed cell death associated with the hypersensitive response (HR), an effect referred to as localized induced resistance or localized induced response (LIR) (Dow et al*.,* 2000).

Mechanism of root colonization: Preparing for the battle with phytopathogens

If a *Pseudomonas* strain cannot adequately compete within the environment of the rhizosphere and colonize the root surface then it will not be an effective BCA. Substantial efforts have been made to identify genes required for key rhizosphere function(s). Given that the rhizosphere is a complex and ever changing environment, it is not surprising that a diverse array of genes have been shown to play an important role in plant root colonization. To date, several genes involved in nutrient acquisition, motility, chemotaxis, adhesion, secretion and stress response have been implicated in the colonization ability of *Pseudomonas* strains.

One system that can play an important role in modulation of host defence responses by pathogens and PGPP is the type-III protein secretion system (TTSS). Pathogens such as *P. syringae* and *P. aeruginosa* use TTSSs to deliver ‘effector’ proteins into the cytoplasm of host cells. TTSS effectors are highly diverse, but their collective function appears to be to render the host more susceptible to infection, and to promote bacterial multiplication in host tissues (He and Jin, 2003). Plants have responded to the threat of bacterial hijacking by evolving surveillance mechanisms that detect the presence and activities of effector proteins. Recognition

of effectors triggers a pre-emptive defense response known as the HR during the early stages of infection, which generally manifests as localized programmed cell death and accumulation of anti-microbial compounds. Effectors that elicit the HR are referred to as Avr (avirulence) proteins. Recognition of Avr is generally conditioned by a single host protein, an R protein (Dangl et al., 1996). The role of TTSSs in rhizobial symbioses appears to be similar to its role in pathogenesis: to modulate host defenses and promote growth in plant tissues. Preston (2004) reported that TTSS genes are present in many plant-colonizing and plant growth-promoting *P. fluorescens* and *P. putida* strains. Current evidence clearly suggests that plant cells can and do receive TTSS-secreted effectors from a wide range of plant-colonizing bacteria, including PGPP. However, further extensive analyses are needed to address the role of TTSSs in the ecology of plant colonizing bacteria (Preston, 2004).

MECHANISMS OF BIOLOGICAL CONTROL OF FLUORESCENT PSEUDOMONADS

According to the definition by Baker and Cook (1974) disease suppressive soils are “soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil”. This phenomenon, although rare, has been well characterized and there is strong evidence that disease suppression is the result of the presence of certain rhizobacteria with antifungal activity. Many biocontrol agents from *P. fluorescens* and closely related species are well characterized for their ability to produce antimicrobial compounds. The biosynthetic pathways involved in their production, as well as their regulation and the signals involved have received extensive attention (Baehler et al., 2006; Dubuis et al., 2007), and these bacteria have become prominent models for analysis of bacterial secondary metabolism. Three major mechanisms have been proposed to explain the suppressive and antagonistic effects of fluorescent pseudomonads. According to one, the pathogen is inhibited by competition for iron, according to the second mechanism, fluorescent pseudomonads inhibit phyto-pathogens by producing secondary metabolites with antibiotic activity, e.g. phenazines, pyrroles, acetylphloro-glucinols and cyanides (Davison, 1986; Défago and Haas, 1990) and thirdly induction of systemic resistance.

Competition for iron in the micro rhizosphere

Fluorescent pseudomonads owe their fluorescence to an extracellular diffusible pigment called pyoverdin (Pvd) or pseudobactin. This pigment has high affinity for Fe^{3+} ions (the association constant of the interaction (*K*ass) is ~1024 at pH 7) and is a siderophore (iron-carrier) of the producer strain (Meyer and Abdallah, 1978). Ferripyoverdin (that is, Pvd complexed with Fe^{3+}) interacts with a specific outer-membrane receptor, which is present in the producer but might also occur in some non-producers. Subsequently, Fe^{3+} is transported into the cytoplasm and reduced to Fe^{2+}. The resulting siderophore hypothesis postulates that PGPR exert their plant growth-promotion activity by depriving pathogens of iron (Adhikari et al., 2013). For example, under greenhouse conditions, *P. putida* strain B10 suppressed *Fusarium* wilt and take-all, but this suppression was lost when the soil was amended with iron, which repressed siderophore production in this strain (Kloepper et al., 1980). A critical assessment of the siderophore hypothesis shows that in some, but not all, plant-pathogen systems tested under various environmental conditions, Pvd-negative (Pvd-) mutants of fluorescent pseudomonads protect plants less effectively than do the parental strains (Keel et al., 1989; Loper and Buyer, 1991). It is important to point out that Pvd-mediated iron deprivation is a contingent biocontrol mechanism, which works much better at pH 8 than at pH 6; this reflects the increasing solubility of Fe^{3+} species with decreasing pH (Elad and Baker, 1985; Misaghi et al., 1988). Another pseudomonad siderophore, pyochelin, has been identified as an antifungal antibiotic in a screening programme (Phoebe et al., 2001). As pyochelin is a relatively weak Fe^{3+} chelator, but a good Cu^{2+} and Zn^{2+} chelator (Cuppels et al., 1987; Visca et al., 1992), it might be able to deprive some fungi of copper and/or zinc. Although siderophores are part of primary metabolism (because iron is an essential element), on occasion they also behave as antibiotics (which are commonly considered to be secondary metabolites).

Role of secondary metabolites in biological control of phytopathogens

Production of antibiotics in several strains of fluorescent pseudomonads has been recognized as a major factor in suppression of root pathogens. A number of disease suppressive antibiotic compounds have been characterized, including N-containing heterocycles such as phenazines, pyrrole-type antibiotics, pyo-compounds and indole derivatives as well as non- N- containing 2,4-diacetylphloroglucinol (DAPG). *In vitro*, these antibiotics inhibit fungal pathogens, but they can also be active against many bacteria and, in some cases, against higher organisms. The natural decline in ‘take-all’ disease (TAD) of wheat root caused by *Gaeummanomyces graminis tritici* (Ggt) during extended monoculture of wheat, is an interesting and extensively studied example of natural

biological control phenomenon and antibiotics are thought to be responsible for the reported biocontrol. The antibiotics pyoluteorin (Plt), pyrrolnitrin (Prn), phenazine-1-carboxylic acid (PCA) and 2,4-di-acetylphloroglucinol (Phl) have drawn great attention of research in biological control, since they help in competition within the rhizosphere milieu.

2, 4-di-acetylphloroglucinol (DAPG or Phl)

A broad-spectrum antibiotic, Phl is a phenolic molecule produced by many fluorescent pseudomonads and exhibits antifungal, antibacterial, antihelmenthic and phytotoxic activities (Abbas et al., 2002). In addition, it shows herbicidal activity resembling 2,4-dichlorophe-noxyacetic acid (2,4-D). Phl is a polyketide synthesized by condensation of three molecules of acetyl CoA with one molecule of malonyl CoA to produce the precursor monoacetylphloroglucinol (MAPG), which is subsequently transacetylated to generate DAPG. This means that various biotic and abiotic factors associated with field location and cropping time affect the performance of fluorescent pseudomonads (Notz et al*.,* 2002). Complex biotic factors such as plant species, plant age, host cultivar and infection with the plant pathogen *Pythium ultimum*, can significantly alter the expression of the gene *phlA* (Notz et al*.,* 2001). Among abiotic factors, carbon sources and various minerals influence production of Phl. Fe^{3+} and sucrose have been reported to increase the levels of DAPG and MAPG in *P. fluorescens* F113, whereas in *P. fluorescens* Pf-5 and CHA0, Phl was stimulated by glucose (Nowak et al*.,* 1994; Duffy and Défago 1999). In *P. fluorescens* strain S272, highest DAPG yield was obtained with ethanol as the sole source of carbon. Microelements, such as Zn^{2+}, Cu^{2+} and Mo^{2+} have been found to stimulate Phl production in *P. fluorescens* CHA0 (Notz et al*.,* 2001). The exact mechanism of DAPG action is still unclear, although it is known that disease suppression by this antifungal molecule is a result of interaction of specific root-associated microorganisms and the pathogen. Phl also appears to cause induced systemic resistance (ISR) in plants.

The genetic constituents of 2, 4-di-acetylphloroglucinol

The sequences of the entire biosynthetic loci of *phl* are now available for *P. fluorescens* strain Q2-87 in the EMBL database (accession no. U41818). A total of five complete open reading frames (ORFs) and one partial ORF, within the 6.8 kb segment of DNA, is responsible for biosynthesis of DAPG. The genes *phlA*, *phlC*, *phlB* and *phlD* are contained in a large transcriptional unit transcribed in the same direction. This is similar to *phlE*, which is located downstream of *phlD* (Delany et al*.,* 2000). The gene *phlE* produces a red pigment that is involved in the transport of Phl out of the cell. In spite of these developments, the precise role of each gene in Phl biosynthesis is not clear. Another divergently transcribed gene, *phlF* is located 421 bp upstream of biosynthetic genes and consists of an ORF of 627 bp with a corresponding protein of 209 amino acids, with predicted molecular mass of 23,570 Da. PhlF is a repressor molecule that exhibits a helix-turn-helix DNA binding motif which regulates the Phl operon (Bangera and Thomashaw, 1996; Delany et al*.,* 2000). PhlF regulates the biosynthesis of Phl at the transcriptional level. The *phlA*-*phlF* intergenic region displays a complex organization wherein *phlA* is transcribed from a *s*32 RNA pol-dependent promoter that overlaps the promoter of the divergently transcribed *phlF* gene. Another specific sequence of 30 bp, known as *phlO*, is located downstream of *phlA*.

Interaction of PhlF repressor protein with this sequence results in repression. This signifies that the repression occurs by inhibition of promoter clearance (Bangera and Thomashaw, 1996; Schnider et al*.,* 1995). Two more regions of 7 and 9 bp are located in the intergenic region of *phlA*-p*hlF*, which not only bind the repressor but further strengthen the binding. The repression however occurs only during the early log phase, after which it is ineffective because of its interaction with the inducer Phl. Salicylate can interact with PhlF to stabilize its interaction with the *phlA* promoter, leading to tighter repression of Phl production. Thus, interactions of these molecules with PhlF contribute towards complex regulation of Phl biosynthesis (Corbell and Loper, 1995).

Phenazines

Phenazines (Phz) are N-containing heterocyclic pigments synthesized by *Brevibacterium*, *Burkholderia*, *Pseudomonas* and *Streptomyces* (Budzikiewicz, 1993; Stevans et al*.,* 1994). The intense colour of this molecule, its antibiotic property and involvement in pathogenic reaction have made it an interesting molecule for study. Currently, over 50 naturally occurring Phz compounds have been described and mixtures of as many as ten different Phz derivatives can occur simultaneously in one organism (Mavrodi et al., 1998). For example, *P. fluorescens* 2-79 produces mainly phenazine 1-carboxylic acid (PCA), whereas *P. aureofaciens* 30-84 not only produces PCA but also lesser amounts of 2-OH-phenazines. The major Phz synthesized by *P. aeruginosa* is pyocyanin (1- OH-5-methyl Phz). Almost all Phz exhibit

broad spectrum antibiotic activity against bacteria and fungi and they exhibit it by inhibiting the electron transport system of the pathogens.

Phz also play an important role in microbial competition in rhizosphere, including survival and competence (Mazzola and Cook, 1992). Phenazine nucleus is formed by the symmetrical condensation of two molecules of chorismic acid wherein the amide nitrogen of glutamine serves as the immediate source of N in the heterocyclic nucleus. PCA is the first Phz formed, which gets converted to PCA and acts as the key intermediate in the synthesis of other Phz in fluorescent pseudomonads (Mann, 1987). The broad-spectrum activity exhibited by Phz compounds against fungi and other bacteria is not well understood. However, it is believed that Phz can accept electrons, yielding a relatively stable anion radical that readily undergoes redox cycle. It includes biosynthesis of Mn-containing superoxide dismutase (MnSOD) which causes enhanced production of - 2O (superoxide radical). There is a distinct possibility that the antibiotic action of pyocyanin is actually a result of toxicity of - 2O and H_2O_2 produced in increased amounts in its presence (Wood and Pierson, 1996).

The genetic constituents of phenazines

Structural and functional analysis shows that seven genes, *phzABCDEFG*, are involved in the synthesis of PCA. These are localized within a 6.8 kb fragment in *P. fluorescens* 2-79 (Mavrodi et al., 1998). The Phz biosynthetic loci in *P. fluorescens* 2-79 (Mavrodi et al., 1998), *P. aeruginosa* PAO1 and *P. chlororaphis* PCL 1394 are highly conserved (Chin-A-Woeng et al., 1998). Each *phz* locus contains a set of seven gene core operons, regulated in a cell density-dependent manner by homologues of LuxI and LuxR (Chin-A-Woeng et al., 1998; Latifi et al., 1995), which are found directly upstream of the Phz core. The core gene products, PhzC, PhzD and PhzE, which are homologous with PhzE, PhzA and PhzB in strain 30-84, are similar to enzymes of shikimic acid and chorismic acid metabolism (Delany et al., 2001). PhzG is similar to pyridoxamine 5′ phosphate oxidase, which was found to be the source of cofactor for the PCA synthesizing enzyme(s).

Products of PhzA and PhzB genes are highly homologous and appear to be involved in the stabilization of a putative PCA-synthesizing multienzyme complex. The conversion of PCA to 2-OH-PCA in strain 30-84 is brought about by a gene *phzO* which is located immediately downstream of the biosynthetic operon in strain 30-84 (Mavrodi et al., 1998). PhzO is a non-heme, flavin diffusible monooxygenase that adds a hydroxyl group to PCA at orthoposition relative to carboxyl group, which results in the synthesis of 2-OH-PCA.

Pyrrolnitrin

Pyrrolnitrin [3-chloro-4-(2′-nitro-3′-chloro-phenyl) pyrrole] is an inhibitor of fungal respiratory chain and thus a broad-spectrum antifungal metabolite produced by many fluorescent and non-fluorescent strains of the genus *Pseudomona*s. It was first described by Arima et al. (1964). This highly active metabolite has been primarily used as a clinical antifungal agent for treatment of skin mycoses against dermatophytic fungi, particularly members of the genus *Trichophyton.* A phenyl pyrrol derivative of Prn has been developed as an agricultural fungicide. Pyrrolnitrin persists actively in the soil for at least 30 days. It does not readily diffuse and is released only after lysis of host bacterial cell. This property of slow release facilitates protection against *Rhizoctonia solani* as the cell dies (Schnider et al., 1995).

The genetic constituents of pyrrolnitrin

The *prn* operon has been completely sequenced; *prnABCD* spans 5.8 kb DNA which encodes Prn biosynthetic pathway in which four ORFs, *prnA*, *prnB*, *prnC* and *prnD* are involved. Two stem-loop structures, which are similar to *s*-independent transcription termination signals, have been identified in this sequence. One gene is located immediately 5′ to the beginning of ORF 1, while the other is located at the end of ORF 4. However, there are no *s*-independent transcription termination signals within or between the four ORFs (Gaffeny et al., 1994). All four ORFs are located on a single transcriptional unit. The four genes encode proteins which are identical in size. Among these, *prnA* gene product catalyses the chlorination of L-trp to 7 chloro-L-trp (Hammer et al., 1997). The *prnC* gene product chlorinates it at the 3-position to form an amino pyrrolnitrin. The *prnD* gene product catalyses the oxidation of aminopyrrolnitrin to a nitro group to form pyrrolnitrin (Kirner et al., 1998). The organization of *prn* genes in the operon is identical to the order in which the reactions are catalysed in the biosynthetic pathway.

Pyoluteorin

Plt is an aromatic polyketide antibiotic consisting of a resorcinol ring, which is derived through polyketide biosynthesis. This in turn is linked to a bichlorinated pyrrole moiety, whose biosynthesis remains unknown (Kitten et al., 1998; Nowak et al., 1999). Biosynthesis of Plt is initiated from proline or amrelated molecule, which condenses serially with threemacetate equivalents coupled to chlorination and oxidation at yet unidentified stages. The formation and cyclization of the C-skeleton

has been reported to proceed by the action of a single multienzyme complex (Nowak et al., 1999). Proline is the primary precursor of dichloropyrrole moiety of Plt.

The genetic constituents of pyoluteorin

Ten genes, *pltLABCDEFG* are involved in the biosynthesis of Plt. They span a 24 kb genomic region in *P. fluorescens* Pf-5. Among these ten genes, *pltB* and *pltC* encode type 1 polyketide synthetase and *plt*G encodes a thio esterase, three halogenases are coded by *pltA*, *pltD* and *pltM*37. Except for a 486 bp gap between the coding regions of *pltL* and *pltR*, contiguous *plt* genes are separated by less than 50 bp.

pltR and *pltM* are transcribed divergently from *pltLABCDEFG* gene cluster; a sequence within 486 bp intergenic region separates *pltRM* from the gene cluster. Among the *plt* gene products, PltR is similar to LysR family of the transcriptional activators (Nowak et al., 1999; Pierson et al., 1998). Furthermore, PltR acts as a positive transcriptional activator linked to loci like *phzI* of the Phz biosynthetic locus. However, signals required for the transcription of *pltR* coinducer are yet to be identified (Chin et al., 2003; Pierson et al., 1998).

Mechanism of regulation of biological control: The ambiguity slowly shown

Three levels of regulation

The regulation of the production of secondary metabolites such as antifungals is operated in bacteria through various mechanisms acting at transcriptional and posttranscriptional levels. An understanding of how biocontrol bacteria regulate the expression of genes involved in the inhibition of pathogens, is a prerequisite for predicting the environmental conditions under which such bacteria are likely to perform optimally. Three levels of regulation have now been suggested; a primary sensing level which is dependent on the surroundings and a secondary or intermediate level that is responsible for regulation of antibiotic biosynthesis with other metabolic processes through global regulation and cellular homeostasis, and a highly specific tertiary level which requires an involvement of regulatory loci that are linked and divergently transcribed from structural genes for antibiotic biosynthetic genes (Abbas et al., 2002; Haas et al., 2000)

Regulation by typical bacterial two-component system

An environmentally regulated, two-component system is Now known to be essential for antibiotic production in various Gram-negative bacteria, including *Pseudomonas* (Meyer et al., 2002). This prokaryotic, two-component regulatory system is a transmembrane protein that functions as a sensory kinase GacS and the cytoplasmic cognate response regulator GacA protein that mediates changes in gene expression in response to sensor signals. As a consequence of interaction with unknown signals, GacS sensor activates GacA response regulator by phosphorylation. The activator GacA, by virtue of its typical C-terminal helix-turn- helix motif, regulates the transcription of the target genes. While the direct GacA targets are not known, GacS/GacA system exerts a positive effect on cell density-dependent gene regulation; the latter is mediated by *N*-acylhomoserine lactone (AHL) in *P. aeruginosa*, *P. syringae* and *P. aureofaciens*. However, a similar system also effectively operates in other Gram-negative bacteria which do not produce AHL, e.g. *P. fluorescens* CHAO; here, GacS/ GacA strictly controls the expression of extracellular products such as exoenzymes, antibiotics and HCN when cells are in idiophase, that is, transition from exponential to stationary phase (Heeb and Haas, 2001).

Regulation by quorum sensing

Bacterial populations in their natural habitats have now been reported to communicate with each other through chemical signals that are released in a cell density-dependent manner. This is referred to as quorum sensing (QS),that is, a minimum cell number, and operates through two broad categories of molecules, viz. amino acids and short peptide hormones commonly involved in Gram-positive bacteria and fatty acid-derivatives such as AHLs, in Gram-negative bacteria. On the root surface, many biofilm forming bacteria are present, where they can attain high population densities and accumulate the concentration of such signal molecules and regulate various physiological processes (Chin et al., 2003). For example, a large family of regulatory systems has now been described that closely matches the LuxI and LuxR proteins of *V. fischeri*. QS relies on the fact that LuxI-type proteins synthesize AHLs (also known as autoinducers), which diffuse from bacteria that produce them either passively or by means of active efflux and accumulate at high population densities. AHL binds to and activates LuxR-type receptor proteins. These function either as cytoplasmic transcriptional factors or as repressors (Zang et al., 2002).

Regulation by sigma factors

Regulation of antifungals operates at another level as well. This requires involvement of sigma factors that are otherwise an integral component of regulation of antifungals like Phl and Plt, e.g. *P. fluorescens* Pf-5. The

overexpression of activator gene *rpoD* or mutation deletion of suppressor gene *rpoS* increases Phl or Plt production (Kitten et al*.,* 1998). These genes encode sigma-factor σ^{32} and stationary-phase σ^{38} respectively, required during transcription. This suggests that σ factors compete for RNA pol, and any imbalance either due to excess of σ^{32} or lack of σ^{38} might enhance the expression of genes; the expression is driven by weak σ^{32}-dependent promoters (Schnider et al*.,* 2000).

Regulation via small RNA binding protein

In *P. fluorescens* CHA0, this system tightly controls the expression of several biocontrol factors including Phl, HCN, pyoluteorin, pyrrolnitrin and exoprotease (Haas and Keel, 2003). The GacS sensor kinase has an autophos-phorylation domain around His294, a phosphoacceptor domain around Asp717 and a histidine phosphotransfer domain around His863. On interaction with bacterial signal molecules, GacS is autophos-phorylated and a phospho-relay mechanism transfers a phosphate residue to the acceptor domain of the response regulator GacA159, 160; this then activates, directly or indirectly, the transcription of the three small RNA genes *rsmX*, *rsmY* and *rsmZ*. Titration of these RNAs by the RsmA and RsmE proteins relieves the translational repression exerted by these proteins at, or near, the ribosome binding site (RBS) of the target mRNAs (for example, *hcn* for HCN synthase, *apr* for exoprotease and *phl* for Phl synthase). Currently, the involvement of GacS/GacA two-component regulatory system in the production of secondary metabolites such as phenazines has been extensively studied for biological control activity in *Pseudomonas chlororaphis* 30-84 (Wang et al*.,* 2013).

Regulation via microbial metabolites

Microbial metabolites also play an important role in the regulation of antifungal molecules, e.g. synthesis of DAPG is autoinduced and repressed by other bacterial extracellular metabolites of strain CHAO (Schnider et al*.,* 2000). Furthermore, salicylate and secondary metabolites (fusaric acid and Pln) have negative effect on Phl production (Abbas et al*.,* 2002). Salicylate interacts with repressor PhlF and stabilizes its interaction with *phl*A promoter. This results in tighter repression of Phl production.

ROLE OF INDUCTION OF SYSTEMIC RESISTANCE IN BIOLOGICAL CONTROL: THE HOST IMMUNIZATION

Induced resistance is a state of enhanced defensive capacity developed by a plant when appropriately stimulated (van Loon et al*.,* 1998). In 1991, two research groups independently described induced systemic resistance (ISR) as the mode of action of disease suppression by nonpathogenic rhizosphere bacteria (Van Peer et al., 1991; Wei et al., 1991). Since then, the involvement of ISR in disease suppression has been studied for a wide range of biological control microorganisms and, in many cases, ISR was found to be involved. Phenotypically, ISR is similar to systemic acquired resistance (SAR) that is triggered by necrotizing pathogens. Although the terms SAR and ISR are synonymous, to distinguish between pathogen- and rhizobacteria-induced resistance, SAR is used for the pathogen-induced type and ISR for the rhizobacteria-induced type. SAR requires accumulation of salicylic acid (SA) in the plant (Sticher et al*.,* 1997), ISR does not and, instead, is dependent on intact responses to ethylene and jasmonic acid (JA) (Pieterse et al*.,* 1998). When these different signal transduction pathways are triggered simultaneously in *Arabidopsis thaliana*, disease suppression is enhanced (Van Wees et al*.,* 2000). This suggests that combining bacterial traits that trigger either the SA, or the ethylene or JA dependent response can improve biological control. To manipulate this phenomenon effectively for practical applications, knowledge on bacterial traits such as flagella, lipopolysaccharides, siderophores, salicylic acid, antibiotics, which are involved in the triggering of ISR is essential.

Bacterial traits that induce systemic resistance

Flagella

Bacterial flagellins, the main protein component of flagella, can elicit defense responses in plants (Gomez-Gomez and Boller, 2000; Zipfel et al*.,* 2004). For *P. putida* strain WCS358, the involvement of flagella in ISR was studied in *Arabidopsis*, bean and tomato by applying isolated flagella and by using non-motile mutants that lack flagella (Meziane, 2005). In *Arabidopsis*, application of WCS358 flagella triggered ISR against *P. syringae* pv. *tomato*, whereas in bean or tomato, their application did not lead to induced resistance.

Lipopolysaccharide

Lipopolysaccharides (LPS) have been implicated in ISR triggered by fluorescent pseudomonads in carnation (Van Peer and Schippers, 1992), radish (Leeman et al*.,* 1995), and *A. thaliana* (Van Wees et al*.,* 1997). In the case of ISR against *Globodera pallida* (Stone) Behrens on potato

by *Rhizobium etli,* the LPS also plays a major role (Reitz et al*.,* 2002). In these studies, either purified LPS was used to induce systemic resistance, or mutants that lack part of the LPS were compared with the parental strain regarding their abilities to induce resistance.

Role of siderophores

Siderophores are low-molecular-weight molecules that are secreted by microorganisms to take up iron from the environment (Höfte, 1993), and their modes of action in suppression of disease were thought to be solely based on competition for iron with the pathogen (Duijff et al*.,* 1999). Interestingly, siderophores can induce systemic resistance (ISR) (Leeman et al*.,* 1996). A clear cut role for siderophores in ISR was reported for *P. putida* WCS358 in suppression of bacterial wilt in *Eucalyptus urophylla*, caused by *Ralstonia solanacearum* (Ran et al*.,* 2005). For *P. fluorescens* WCS374, the situation was different in that wild type bacteria, the pseudobactin mutant, and the purified pseudobactin were all effective in suppression of the disease, indicating redundancy of ISR-triggering traits of WCS374 in this system. Pseudobactin mediated ISR was also found to be effective against *Tobacco necrosis virus* in tobacco. A pseudobactin-minus mutant of *P. fluorescens* CHA0 was less effective in reducing numbers of viral lesions and lesion diameter than the parental strain (Maurhofer et al., 1994).

Salicylic acid

A rhizobacterial metabolite that was suggested to trigger the SA-dependent signal transduction pathway is SA itself (De Meyer and Höfte, 1997; Maurhofer et al*.,* 1998). Exogenous application of SA, even at the extremely low dose of 100 fg, to radish roots significantly reduced Fusarium wilt (Leeman et al*.,* 1996). Salicylic acid production has been observed for several bacterial strains, and exogenously applied SA can induce resistance in many plant species. For example, *P. fluorescens* strain WCS374 produces relatively large quantities of SA under conditions of iron limitation. Moreover, WCS374 is more effective, in radish, in ISR against fusarium wilt under iron-limited conditions when compared with conditions of sufficient iron availability (Leeman et al*.,* 1995). Possibly, the iron-regulated ISR by WCS374 in radish is mediated by the SA-containing siderophore pseudomonine (Mercado-Blanco et al*.,* 2001). Thus, for several bacterial strains, a role of bacterially produced SA in induced resistance has been suggested, but upon careful examination, SA seems not to be involved directly (Audenaert et al*.,* 2002).

Role of antibiotics

Production of antibiotics has been described as a powerful mode of action in disease suppression by which development and (or) activity of the pathogen is thought to be directly inhibited (Handelsman and Stabb, 1996). A role for DAPG in ISR was recently demonstrated in *Arabidopsis.* In this plant system, DAPG produced by *P. fluorescens* CHA0 is the key compound in ISR against *Peronospora parasitica* (Iavicoli et al*.,* 2003). Also in *Arabidopsis*, ISR against *P. syringae* pv. tomato by *P. fluorescens* Q2-87 depends on the production of DAPG (Weller et al*.,* 2004). In tomato, *P. fluorescens* CHA0 induces resistance against the root-knot nematode *Meloidogyne javanica*. Also, in this case, DAPG appears to be the bacterial trigger of ISR, since a DAPG-minus mutant was not effective and effectiveness was restored by complementation of the mutant (Siddiqui and Shoukat, 2003). Finally, in *P. aeruginosa* 7NSK2 the phenazine antibiotic pyocyanin is involved in ISR against *B. cinerea* in tomato (Audenaert et al*.,* 2002).

Role of β-aminobutyric acid

Currently, β-aminobutyric acid (BABA), non-protein amino acid, is thought to be important component of the signaling pathway regulating ISR response in plants. Knowledge about how the resistance is induced by BABA against biotic factors is still not fully understood. In the first step of defense, the plant produces reactive oxygen species (hypersensitivity response), and induces physical barrier by callose deposition and lignin accumulation in the cell walls. In addition, biochemical mechanisms of response to the stress are also initiated, for example biosynthesis of secondary metabolites (phenols, anthocyanin, phytoalexins) and induction activity of enzymes associated with active oxygen species, lignifications and plant secondary metabolism (Justyna and Ewa, 2013).

Role of population density

In a dose-response study of ISR mediated by *P. fluorescens* WCS374 in radish, it was revealed that population densities of 105 colony forming units per gram of root are required for significant suppression of disease (Raaijmakers et al*.,* 1995). In situations where no bacteria are introduced, population densities of one single bacterial genotype probably never reach such high densities, making it unlikely that, in the field, plants are triggered sufficiently by bacteria to express ISR. In dose-response studies, it was demonstrated that RS111a was effective in suppressing fusarium wilt of both radish and

flax at much lower initial population densities than RS111. These results suggest that the level of disease control through ISR by a specific strain can be improved. Further studies to elucidate the determinants of ISR of these strains are ongoing.

Molecular mechanism of SAR and ISR

Early research on molecular mechanism involved in pathogen induced SAR showed that the onset of SAR is accompanied by a local and systemic increase in the endogenous levels of salicylic acid (Malamy et al*.,* 1990) and the concomitant up-regulation of a large set of genes (Ward et al*.,* 1991) including the pathogenesis related (PR) proteins (Van loon and Van strien 1999). Several PR proteins possess antimicrobial activity and are thought to contribute to the state of resistance attained. Genetic screens for SAR compromised Arabidopsis mutants revealed a series of mutants that all appeared to be affected in the same gene (Delaney et al*.,* 1995), the npr1 (non-expresser of PR genes) or nm1 (non-immunity). Mutant *npr1* plants accumulate normal levels of salicylic acid after pathogen infection, but are impaired in their ability to express PR genes and to mount SAR response, indicating that NPR1 (the gene product of *npr1*) functions downstream of SA in the SAR pathway. Recently, it has been demonstrated that, upon induction of SAR, NPR1 which possess ankyrin-like repeats, is translocated to the nucleus, where it activates the PR gene expression by physically interacting with a subclass of basic leucine zipper protein transcription factors (TGA transcription factors) that bind to promoter sequences required for SA- induced PR gene expression. Recent study has shown that the *Arabidopsis* NPR1 (nonexpresser of *PR* genes 1) protein is a master regulator of SAR. Salicylic acid directly binds to the NPR1 adaptor proteins NPR3 andNPR4, regulates their interactions withNPR1, and controls NPR1 protein stability. However, how NPR1 interacts with TGA transcription factors to activate defense gene expression is still not well understood. In addition, redox regulators, the mediator complex, WRKY transcription factors, endoplasmic reticulum-resident proteins, and DNA repair proteins play critical roles in SAR. SAR can even be passed on to progeny through epigenetic regulation (Fu and Dong, 2013).

Besides SA, the plant growth regulators jasmonic acid (JA) and ethylene (ET) have repeatedly been implicated in the regulation of primary resistance responses in plants (Pieterse et al*.,* 2001). Exogenous application of these compounds often results in an enhanced level of resistance. To investigate the role of JA and ET in rhizobactria mediated ISR the Arabidopsis JA response mutant jar1-1 and the ET response mutant etr1-1 were tested for their ability to express ISR. Both mutant were unable to mount resistance against Pst DC 3000 after colonization of the roots by *P. fluorescens* WCS417r (Pieterse et al., 1998), indicating that ISR requires responsiveness to both JA and ET. Furthermore, in the study of Pieterse et al. (1998) it was postulated that JA and ET induced ISR was SA- independent pathway and follows a signaling pathway in which components from the JA and ET response are successively engaged. Previously in several studies it was documented that NPR1 is an important regulatory factor in the SA dependant SAR response.

In 1998, Pieterse et al. demonstrated with Arabidopsis mutant npr1, that, like pathogen induced SAR, rhizobacteria mediated ISR is an NPR1 dependant defense response. Elucidation of the sequence of ISR signaling events revealed that NPR1 functions downstream of JA and ET in the ISR signaling pathway. NPR1 is able to differentially regulate defense genes expression depending on the signaling pathway that is activated upstream of it. In the year 2002, to study the association between induced resistance and basal resistance, the research group of Ton et al. (2002) used a collection of 11 Arabidopsis eds mutants with enhanced disease susceptibility (reduced basal resistance). Through this study they successfully demonstrated that EDS4 (product of eds4-1) for ET and EDS8 (product of eds8-1) for JA plays an important role downstream of JA and ET signaling pathway. Future research should reveal the exact role of these signaling components in the expression of ISR.

BIO CONTROL IN PRACTICE: THE PRACTICAL SCENARIO

This is the most important contexts in which biological control mediated by *P. fluorescens* strains and related pseudomonads has important practical implications. The first context corresponds to the use of biocontrol agents as inoculants of soil or plants, which has been successfully implemented in agronomic field trials (Amein et al*.,* 2008; Karthikeyan and Gnanamanickam, 2008). The use of P. fluorescens biocontrol agents is thought to have a limited ecological impact on indigenous saprophytic populations and to take place without negative side-effects on rhizosphere functioning (Mark et al*.,* 2006).

The principal reasons of inconsistency of biocontrol products under field condition

Although the vast body of research on *Pseudomonas* BCAs deals with their capacity to control soil-borne fungal

pathogens, there has been limited success developing commercially viable products.

Depletion of cell count

Many inoculation products are commercially available (Mark et al*.,* 2006), but strains of *Pseudomonas* spp. can mutate in culture and generally lose viability when stored for a period of several weeks (Haas and Défago, 2005). Any mutation or lack of viability may greatly affect performance and could be a catastrophic occurrence for the company selling the product. Efficient quality control will be definitely essential. Reliable assays to check the efficacy of the strains will be necessary. Methods for monitoring inoculants quality are being developed. Recent advances show that pseudomonas formulation can be improved for long term storage (Guo et al*.,* 2004) and efficient antagonistic activity (Wiyono et al*.,* 2008).

Root colonization

In addition, pseudomonas inoculants may perform inconsistently from one field to another and/or from one year to the next, as a consequence of variability in root colonization (Weller, 2007) or in expression of biocontrol traits (Mark et al*.,* 2006). Therefore, superior root colonization and effective functioning in the rhizosphere are key criteria when selecting strains, and research aims at better understanding the molecular basis of these traits (Mavrodi et al*.,* 2006) and the signaling processes regulating the ecology of *P. fluorescens in situ* (Dubuis et al*.,* 2007; Barret et al*.,* 2009).

Improving the biocontrol efficacy of pseudomonas biological control agents

Nowadays, many trading products for plant disease control are based on BCAs. Commercial development of this agricultural product must follow several criteria: large application on major crops, quality control, chemical effects and inoculums formulation. Product safety, production costs, effectiveness against target organisms and acreage and value of crops to be treated must all be considered in the development of biological control and PGPR.

Genetic modification to produce enhanced secondary metabolites

One strategy to develop improved biocontrol strains is to use genetic modification to enhance this activity. To a certain extent, this involves the construction of strains that produce increased levels of antimicrobial metabolites. More significant, however, is the recent focus on developing strains in which the timing of production is altered. This is crucial because, in general, *Pseudomonas* spp. only produce antifungal metabolites at high cell densities during the late logarithmic or stationary phase of growth. If the relevant biosynthetic genes are uncoupled from their regulatory controls, however, this may facilitate early production of antifungal metabolites, offering immediate protection to crop seeds/seedlings.

Control of gene expression at the transcriptional level is recognized as a primary mechanism for modulating the production of secondary metabolites. In this regard, the use of alternative σ factors has received considerable attention. Specific transcriptional activators/repressors may also regulate the transcription of secondary metabolite biosynthetic genes, for example, the PhlF protein, which is expressed from the Phl locus, represses transcription of the *PhlA-D* operon, which comprises genes encoding proteins that direct the synthesis of Phl (Delany et al*.,* 2000, Schnider et al*.,* 2000).

Mutation of *phlF* in a *P. fluorescens* strain increased Phl production *in vitro* during the early logarithmic phase of growth. Similarly, overexpression of *phlA-D* resulted in Phl overproduction and, concomitantly, enhanced biocontrol efficacy against *P. ultimum* in laboratory microcosm trials (Delaney et al*.,* 2001).

Enhancement of rhizosphere competence

Other studies have focused on the possibility of promoting microevolution of biocontrol strains to enhance their rhizosphere competence (de Weert et al*.,* 2004). Promising results were also obtained with the development of genetically-improved strains with higher plant protection ability, either by reprogramming the regulation of existing biocontrol traits (Mark et al*.,* 2006) or the introduction of novel mechanisms such as the degradation of pathogen quorum-sensing molecules (Molina et al*.,* 2003) or ACC deaminase activity (Wang et al*.,* 2000).

Sustainability at the crop micro-rhizosphere environment

The identification of factors that control the fate and performance of the inoculants, small-scale analyses are needed due to the heterogeneity characterizing the complex soil and rhizosphere environments (Sørensen et al*.,* 2001). Direct staining techniques and advanced microscopy had provided the first detailed single cell

images of root colonization by these bacteria using fluorescent antibodies, fluorescent *in situ* hybridization and marker gene technology. Fluorescence-labeled antibodies have been used with success for detection of root-colonizing *Pseudomonas* strains by immune-fluorescence microscopy (Kragelund and Nybroe, 1996, Troxler et al*.,* 1997). The rRNAtargeted fluorescent *in situ* hybridization (FISH) technique offers the possibility of non-extractive detection of target bacteria. More detailed *in situ* studies of single-cell distribution of *Pseudomonas* inoculants, using immunochemical methods and/or FISH have only been possible with the advent of confocal laser scanning microscopy (CLSM) (Lübeck et al*.,* 2000). Insertion of marker or report genes has been commonly used to distinguish the introduced pseudomonads from indigenous populations (Götz et al*.,* 2006). Some of the most popular genes used as markers or reporters encode enzymes are *luxAB, lacZ, luc, xylE* and *gusA* in which case a product of the reaction catalyzed by the marker gene is monitored (bioluminescence, color, etc.) (Sørensen et al., 2001). Gene product can also be detected by immunochemical techniques. Specific physical properties of the gene products may mediate their detection. It occurs to the product of *gfp* gene that is a protein, which emits green fluorescence when excited (Chalfie et al*.,* 1994).

Application of consortia

Another way to seek more effective biocontrol treatments is to inoculate consortia of *P. fluorescens* biocontrol agents, sometimes in mixture with other plant-beneficial microbes (Karthikeyan and Gnanamanickam, 2008). However, the compatibility of these inoculants despite possible bacteriocin-mediated competition (Validov et al*.,* 2005), their antimicrobial metabolites and their extracellular signals needs to be considered (Molina et al*.,* 2003; Dubuis et al*.,* 2007), especially when synergistic effects are sought. Recently, in a study conducted by Antonelli et al*.,* (2013) it was shown show that protection of melon against *Monosporascus cannonballus* has been successfully achieved by applying a consortia of *Bacillus sp.* BsCR and *Pseudomonas putida* PpF4.

Compatibility with residue chemicals

Another important point to be considered is the pesticide use in the cropping systems. Apparently the majority of fungicides do not affect PGPR. Fungicides used on potato seed pieces, like benomyl, captan, PCNB did not affect PGPR strains but mancozeb was inhibitory using *in vitro* tests. *Pseudomonas* spp. Have a biochemical machinery that confers many possibilities to degrade many compounds (Kononova and Nesmeyanova, 2002; Spiers et al*.,* 2000). However, further test will be necessary as new strains are found and new crops with various chemical needs are tested.

Development of bio-control formulation

An equally important, if not over-riding bottleneck, however, is the lack of suitable inoculants formulations that allow *Pseudomonas* cells to survive for long periods under storage at concentrations high enough to afford biocontrol (McQuilken et al*.,* 1998). Initially, bacterization of seeds utilized bacterial growth from culture media suspended in water. Although growth responses were noted in some cases, the problems with this system on a large scale are apparent. Strains may soon die in water suspensions and unless planting are done immediately after treatment, the inoculum dries and the populations of PGPR decline (Rainey, 1999). Powered formulations of PGPR have been developed and a number of commercially available gums were tested as suitable substrates for PGPR in comparison with methylcellulose. It was observed that PGPR establishment on roots and stimulation growth depends on the carrier. There is variability between crops that will be encountered when attempting to develop formulations for wide usage. Suslow and Schroth (1982) showed methylcellulose powder formulations were most suitable for pelleting onto sugar-beet. Powered formulations have benefit as the ease of storage, transport and handling. Also, by pelleting seed with a powder formulation is possible to concentrate a higher population of PGPR around the seed than by dipping in bacterial suspensions. The powder formulations also allow for storage for prolonged periods of time until planting. More studies are necessary to determine most efficient forms to apply PGPR to the plants. Furthermore, carbon sources and minerals have been shown to have an important role in antifungal metabolite production by *Pseudomonas* BCAs, suggesting that nutrient amendments to formulations may also be a useful strategy for improving biocontrol efficacy (Duffy and Défago, 1999). Without doubt, however, further research is required on the development and optimization of microbial inoculants formulations, which will be compatible with current seed coating technologies. Furthermore, because survival during seed coating/pelleting and during storage at ambient temperatures is critical for the development of microbial inoculant products, it seems logical that these traits should form an integral part of any screening process for the selection of new *Pseudomonas* BCAs. In India, bio control market is regulated by Central Insecticide Board (CIB), under the section 9(3B), Insecticide act, 1968. A

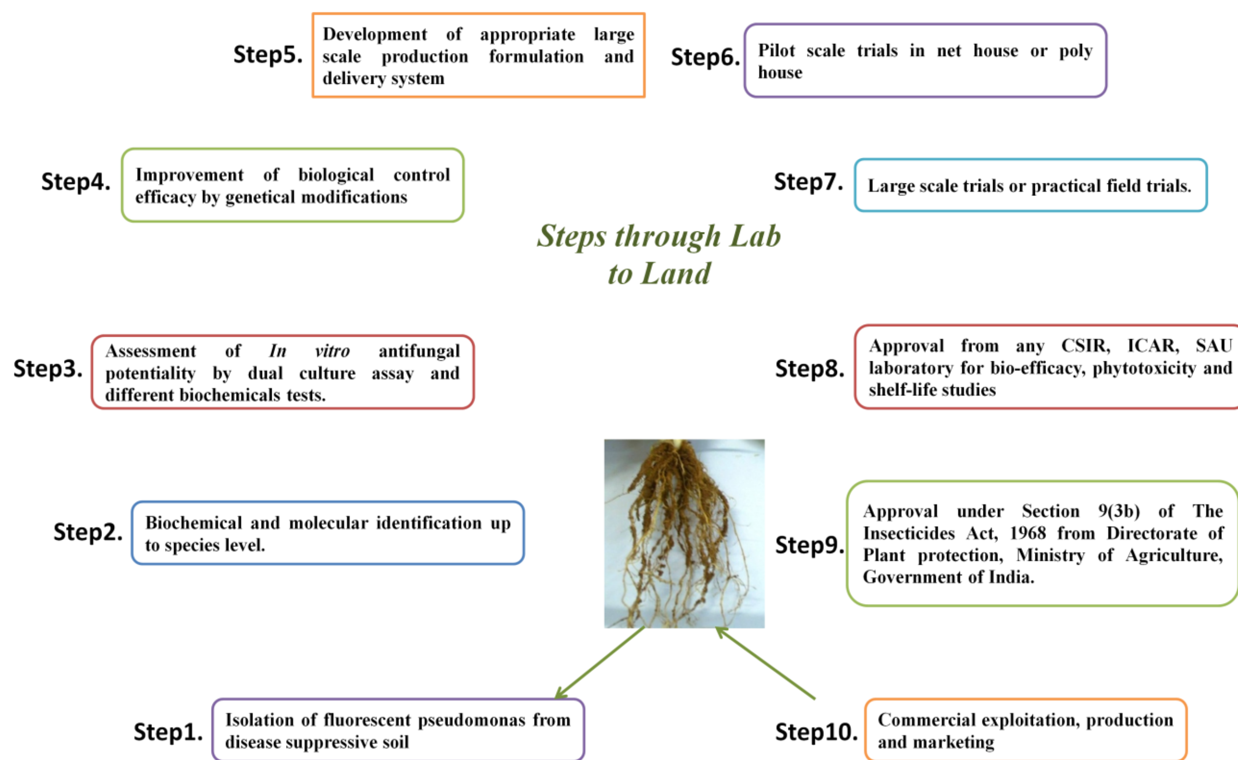

Figure 1. Schematic diagram showing the sequence of events from the isolation of fluorescent *Pseudomonas* from disease suppressive soils, through their development and improvement to their marketing as bio-control products in Indian markets.

schematic diagrammatic representation is given to understand how a potential bio control agent can be marketed in Indian agriculture (Figure 1)

CONCLUSION

Despite a century long history of rhizosphere research we are still at the beginning of understanding the complex plant-microbe interactions in this dynamic environment. The use of fluorescent pseudomonads as biological control agents (BCA) requires the precise understanding of the interactions between plant-bacteria, among bacteria-microbiota and how biotic and abiotic factors influence this relationship.

Exciting developments that will enable in-depth studies of the functioning of the rhizosphere microbiome include the use of phylo-chips to study the composition of microbial communities, development of metatranscriptomics and metabolic profiling of root exudates. In a few years, modern technologies, such as immunofluorescence microscopy, confocal laser scanning microscopy and reporter genes, have improved the study of *Pseudomonas* inoculants in soil and have markedly enhanced the knowledge about their behavior in this environment. Recent advances in studies of the intraspecies and interspecies signaling, quoram sensing are providing an important area for scientific research, as well as, relevant application. In recent years, many strategies to achieve the control of plant diseases using fluorescent pseudomonads as biological control agents (BCAs) have produced significant success, which are relevant since one of the goals of using BCAs, is to make them trustable and assessable product to the farmers. Consequently, continuous searching for new approaches to improve the field efficiency and delivery system of fluorescent pseudomonads as BCAs are strongly required to enable sensible applications to control diseases in a sustainable manner.

Conflict of interests

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

ACKNOWLEDGEMENTS

We, hereby, express our sincere thanks to colleagues at the AICRP on Vegetable Crops, Directorate of Research,

B.C.K.V., Kalyani, West Bengal, India.

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academicJournals

Vol. 8(15), pp. 1562-1572, 9 April, 2014
DOI: 10.5897/AJMR2013.6541
Article Number: 51E450543811
ISSN 1996-0808
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http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Review

Beneficial properties, colonization, establishment and molecular diversity of endophytic bacteria in legumes and non legumes

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Received 30 November, 2013; Accepted 26 February, 2014

Different genera of bacteria are present as endophytic in roots and nodules of legumes and roots of non legumes grown in different parts of the world. A number of these endophytic inhabitants vary from few to large numbers. Predominantly, endophytes belongs to three major phyla, Actinobacteria, Proteobacteria and Firmicutes and includes members of *Streptobyces*, *Azocareus*, *Gluconobacter*, *Pseudomonas, Serratia*, *Stenophomonas*, *Bacillus*, *Paenibacillus* and *Enterobacter*. Majority of the endophytes show plant growth promotion and have other beneficial traits like enhancement of biological nitrogen fixation, phytohormone production, phosphate solubilization, inhibition of ethylene biosynthesis in response to biotic or abiotic stress and above all have biocontrol activity. These endophytes colonize inside the root or nodules. It seems no host specificity exist between different endophytic bacteria and plant host. These opportunist bacteria can enter the plant tissue whenever they have the opportunity either after dissolving the cell wall or through crack entry. Beneficial effects of bacterization of these endophytes have been shown in different plant host under green house and even under field conditions. Therefore endophytic bacteria are better alternate to sustain crop productivity.

Key words: Nodules, endophytic bacteria, legumes, roots, genera.

INTRODUCTION

The plant root defines the interface between a multi cellular eukaryote and soil, one of the richest microbial ecosystems on Earth. Notably, soil bacteria are able to multiply outside and inside roots and modulate plant growth and development (Bulgarelli et al., 2012). Plants are constantly involved in interactions with a wide range of bacteria. These plant associated bacteria colonize the rhizosphere (rhizobacteria), the rhizoplane (epiphytes) and the inside of plant tissues (endophytes). Endophytes are sheltered from environmental stresses and microbial competition by the host plant and they seem to be ubiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots and seeds of various plant species (Kobayashi and Palumbo, 2000). The term

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'endophytic bacteria' is referred to those bacteria, which colonizes in the interior of the plant parts, viz, root, stem or seeds without causing any harmful effect on host plant (Hallmann and Berg., 2006; Ryan et al., 2008).

Symbiotic interactions are the driving force in ecosystems; symbiosis ranges from parasitism to mutualism and includes everything in between. The fitness outcomes for plants differ accordingly: if a plant is highly susceptible to pathogens, its fitness is likely to be low in pathogen rich environments; if a plant cooperates with mutualists, it is likely to thrive even in adverse environments. Bacteria, which colonize the interface between living plant roots and soil, namely the rhizosphere, are abundant symbiotic partners of plants. These so called rhizobacteria are said to be plant growth promoting (PGP). Although all the approximately 300,000 plant species have been estimated to harbor one or more endophytes, few relationships between plants and these endophytes have been studied in detail; the legume-rhizobia symbiosis is an exception (Strobel et al., 2004). The mutualistic interaction of legumes with rhizobia involves finely tuned recognition steps which ultimately lead to the production of root nodules in which the plants accommodate the bacteria (Oldroyd et al., 2005). For other endophytic rhizobacteria, the processes of host-microbe signaling and colonization, and the mechanisms leading to mutual benefit are less well characterized.

In recent years, interest in endophytic micro organisms has increased, as they play a key role in agricultural environment and are promising because of their potential use in sustainable agriculture. Endophytes can also be beneficial to their host by producing a range of natural products that could be used in medicine, agriculture and industry (Ruby and Raghunath, 2011). There is increasing interest in developing the biofertilizers for enhancing crop productivity (Saini et al., 2013a). A challenge is posed for systematic optimization for the application of suitable endophytic isolates and the amount of fertilizer to be added to obtain maximum output. One of the major challenges includes selection of plant genotype and age and compatible associative bacteria. Understanding this compatibility would help to enhance productivity by using specific strain for inoculation. Since, the colonization of associative bacteria also depends upon seasonal changes and soil hydric stress, multiple field trials are required to optimize parameters for obtaining the maximum output. Another factor which is to be studied in details is the plant defense response which may limit or reduce the colonization of associative bacteria. In addition, colonization mechanism is still not well understood. Intelligent analysis of genomic and functional genomics studies can help manipulate the conditions to enhance colonization process and increased plant growth properties. Extensive and intensive research on the understanding of associative and endophytic ecology will be major determinant to maximize benefit from these bacteria. Keeping these points in mind, the present status of these aspects is being reviewed.

ENDOPHYTIC MICROBES IN DIFFERENT HOSTS

Endophytic bacteria have been found in almost every plant studied (Ryan et al., 2008). Since the first reliable reports on the isolation of endophytic bacteria from surface sterilized plants (Samish and Etinger-Tulczynska, 1963; Mundt and Hinkle, 1976) more than 200 bacterial genera from 16 phyla have been reported as endophytes. These include both culturable and unculturable bacteria belonging to Acidobacteria, Actinobacteria, Aquificae, Bacteroidetes, Cholorobi, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Firmicutes, Fusobacteria, Gemmatimonadetes, Nitrospira, Planctomycetes, Proteobacteria, Spirochaetes and Verrucomicrobiae (Sun et al., 2008; Mengoni et al., 2009; Manter et al., 2010; Sessitsch et al., 2012). However, the most predominant and studied endophytes belong to three major phyla (Actinobacteria, Proteobacteria and Firmicutes) and include members of *Streptomyces* (Suzuki et al., 2005), *Azoarcus* (Krause et al., 2006), *Gluconobacter* (Bertalan et al., 2009), *Pseudomonas*, *Serratia* (Taghavi et al., 2009), *Stenotrophomonas* (Ryan et al., 2008), *Bacillus* (Deng et al., 2011; Kumar et al., 2013; Saini et al., 2013b), *Enterobacter* (Taghavi et al., 2010) and *Burkholderia* (Weilharter et al., 2011). Saini et al. (2013a) isolated a total of 166 endophytic bacteria from root of legumes, chickpea (*Cicer arietinum*), pea (*Pisum sativum*), and lucerne (*Medicago sativa*), non-legumes wheat (*Triticum aestivum*), oat (*Avena sativa*) and also from nodules of chickpea.

Occurance of endophytes in legumes

The population density of endophytes is highly variable, depending mainly on the bacterial species and host genotypes and also on the host developmental stage, inoculum density, and environmental conditions (Pillay and Nowak, 1997; Tan et al., 2006). There are many reports on occurrence of endophytic bacteria from roots and nodules of legume plants such as alfalfa, bean, chickpea, clover, cowpea, pea, peanut, soyabean, *Acacia, Argyrolobium, Conzattia,* fenugreek, *Hedysarum, Kennedia, Leucaena, Lotus,* mungbean, *Mimosa, Medicago, Melilotus*, *Ornithopus, Onobrychis*, *Oxytropis*, *Psoralea, Scorpiurus, Sesbania, Tetragonolobus* and *Vicia* (Muresu et al., 2008; Dudeja et al., 2012, Dudeja and Nidhi 2014). Bacteria isolated from legume tissues include *Bacillus, Paracoccus, Sphingomonas, Inquilinus, Pseudomonas, Serratia, Mycobacterium, Nocardia, Brevibacillus, Staphylococcus, Lysinibacillus, Bosea,*

Rhodopseudomonas, Phyllobacterium, Ochrobactrum, Starkeya, Agromyces, Ornithinicoccus, Actinobacterium, Paenibacillus, Methylobacterium, Pedobacter, Aerococcus, Stenotrophomonas, Streptomyces, Dyella and others. Endophytic bacteria in a single plant host are not restricted to a single species but comprise several genera and species. It seems that the bacteria that best adapt to living inside plants are naturally selected. Endophytes are recruited out of a large pool of soil or rhizospheric species and clones.

Root associated 72 endophytic bacteria from peanut plants grown in the main producing areas of six provinces in China were isolated (Wang et al., 2013). The 16S rRNA gene sequences and phylogenetic analysis revealed that 49 isolates belonged to Bacilli and 23 isolates to Gammaproteobacteria. Similarly, 39 endophytic bacterial strains were isolated from the nodule of *Lespedeza* sp. grown in two different locations of South Korea (Palaniappan et al., 2010). The strains were identified by using 16S rRNA gene sequence as *Arthrobacter, Bacillus, Bradyrhizobium, Burkholderia, Dyella, Methylobacterium, Microbacterium, Rhizobium* and *Staphylococcus.*

Various rhizobial and non-rhizobial strains were isolated from root nodules of two widespread south eastern Australian tree legumes, *Acacia salicina*, *Acacia stenophylla.* This legume was nodulated primarily by *Bradyrhizobium*, while the results indicate significant associations with other root nodule forming bacterial genera, including *Rhizobium*, *Ensifer*, *Mesorhizobium*, *Burkholderia*, *Phyllobacterium* and *Devosia* (Hoque et al., 2011).

However in the majority, *Streptomyces* was present in Stemona earthnet samples (Wei and Wu, 2012). Endophytic bacteria from roots and nodules of fieldpea and chickpea being grown in Northern India were isolated. A total of 75 endophytic bacteria roots and nodules of fieldpea (Narula et al., 2013a) and 88 from roots and nodules of chickpea showed that 50% in roots and 93.4% in nodules were Gram positive and most of the isolates were spore formers (Saini et al., 2013b). More number of bacteria were present in nodules as compared to the roots of fieldpea as well as chickpea. The endophytic strain Zong1 isolated from root nodules of the legume *Sophora alopecuroides* was closely related to *Pseudomonas chlororaphis* (Zhao et al., 2013). Tagging with *gfp* gene indicated that strain Zong1 may colonize in root or root nodules.

Co-inoculation with Zong1 and SQ1 (*Mesorhizobium* sp.) showed significant effects as compared to single inoculation for siderophore production, phosphate solubilization, organic acid production, IAA production and antifungal activity *in vitro.* These results suggest that *P. chloroaphis* Zong1 and *Mesorhizobium* sp. SQ1 have better synergistic or additive effect and growth index in growth assays under greenhouse conditions is higher than those of single inoculation.

Occurrence of endophytes in non-legumes

There are many examples of reported microbial endophytes and plants harbouring them, including rice, banana, wheat, sugarcane, carrot, maize, potato, coeffee, oats and citrus plants (Sturz et al., 2000; Rosenblueth and Martínez-Romero, 2006; Kumar et al., 2013). Population of endophytes from different tissues of a plant was different qualitatively and quantitatively (Qiao et al., 2006). Both Gram positive and negative bacteria were isolated, with a greater percentage (68%) being Gram negative.

The highest number of bacteria among the berry tissues sampled was isolated from the seed, and includes *Bacillus*, *Burkholderia*, *Clavibacter*, *Curtobacterium*, *Escherichia*, *Micrococcus*, *Pantoea*, *Pseudomonas*, *Serratia* and *Stenotrophomonas* (Vega et al., 2005). Inside the roots and stems of sugarcane plants, genera of *Burkholderia, Pantoea, Pseudomonas* and *Microbacterium* were present (Mendes et al., 2007). A total of 192 bacterial endophytes from roots of rice (*Oryza sativa*) formed 52 operational taxonomic units based on the similarity of the ARDRA banding profiles (Marquez-Santacruz et al., 2010); *Streptomyces* sp. was isolated from roots of a Thai jasmine rice plant (*Oryza sativa* L. cv. KDML105) (Rungin et al., 2012).

Rice endophytic bacteria were identified as two species of *Penibacillus*, three species of *Microbacterium*, three *Bacillus* species, and four species of *Klebsiella* (Jia et al., 2014). Rice seeds treated with the plant growth-promoting bacteria (PGPB) showed improved plant growth, increased height and dry weight and antagonistic effect against fungal pathogens. Berseem clover (*Trifolium alexandrinum*) endophytic bacteria for rice plant growth-promotion were selected on the basis of root colonization bioassay and a plant growth promoting trait (Etesami et al., 2013).

A significant relationship among indole-3-acetic acid producing isolates, the size of root colonization, and plant growth was observed. Endophytic bacteria from *Panax notoginseng* exhibited antagonistic properties against pathogens and all the 104 antagonistic bacteria belong to four clusters: Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes/Chlorobi.

Members of the Firmicutes, in particular the *Bacillus* sp., were predominant in all analyzed tissues (Ma et al., in 2013). All these studies indicated that wide range of microbes exist as endophytic in roots and nodules of legumes and roots of non legumes. It seems that no host specificity of endophyte is existing as entry of a particular bacterial genera in roots or nodules, dependent upon availability of microflora in that particular soil which may be affected by soil and environmental conditions.

BENEFICIAL CHARACTERISTICS OF BACTERIAL ENDOPHYTES

Endophytes play crucial role in plant growth promotion by having beneficial impact on host plant. These bacteria may promote plant growth in terms of increased germination rates, biomass, leaf area, chlorophyll content, nitrogen content, protein content, hydraulic activity, root and shoot length, yield and tolerance to abiotic stresses like drought, flood, salinity, etc. Plant associated bacteria can promote plant growth directly through biological nitrogen fixation, phytohormone production, phosphate solubilization, inhibition of ethylene biosynthesis in response to biotic or abiotic stress (induced systemic tolerance) etc., or indirectly by inducing resistance to pathogen (Bhattacharya and Jha, 2012). Bacterial endophytes have diverse positive effects on their hosts. Some examples include nitrogen fixation, antibacterial and antifungal activities, as well as plant growth promotion (Tan et al., 2006; Rijavec et al., 2007). Other roles such as synthesis of novel chemicals, resistance to heavy metals and xenobiotic degradation have been observed in endophytes (Siciliano et al., 2001). Jha et al. (2013) reviewed plant growth promoting abilities of rhizospheric and endophytic bacteria and their molecular aspects. Plant growth promoting bacteria has been classified on the basis of basic mechanisms through which they stimulates plant growth as PGPB, which induces plant growth directly and; biocontroller, which protects plants by inhibiting growth of pathogen and/or insect (Backman and Sikora, 2008). The different beneficial characteristics of different endophytes reported (Faria et al., 2013) are being discussed here.

Phytohormone production and root growth promotion by endophytes

Plant hormones are chemical messengers that affect a plant's ability to respond to its environment. There are five major groups of hormones: auxins, gibberellins, ethylene, cytokinins, and abscisic acid. Indole-3-acetic acid (IAA) is a member of the group of phytohormones and is generally considered the most important native auxin (Ashrafuzzaman et al., 2009). It functions as an important signal molecule in the regulation of plant development including organogenesis (root growth), tropic responses, cellular responses such as cell expansion, division, differentiation, and gene regulation (Ryu and Patten, 2008). The production of auxin like compounds increases seed production and germination along with increased shoot growth and tillering (Kevin, 2003). Hung and Annapurna (2004) isolated 65 bacterial endophytes from stem, root and nodule of two soyabean varieties, *Glycine max* and *Glycine soja* and 56 isolates were capable of producing IAA in different concentrations. Similarly, a total of 28 endophytic bacteria were isolated from *Sophora alopecuroides* root nodules and one endophytic isolate along with *Mesorhizobium* produced good amount of IAA (Zhao et al., 2011).

Root growth promotion assay is a good parameter to find out whether the endophytes are producing phytohormones or not. A total of 166 endophytic bacteria from roots of legumes, chickpea (*Cicer arietinum*), pea (*Pisum sativum*), and lucerne (*Medicago sativa*) and non-legumes wheat (*Triticum aestivum*) and oat (*Avena sativa*) and nodules of chickpea were isolated. Majority of the endophytes were found to promote the growth of chickpea roots in root growth promotion assay in agar plates, however chickpea nodule endophytic bacteria were better root growth promoters as compared to others (Saini et al., 2013a). Similarly, field pea root growth promotion assay showed that 63.3% nodule endophytic bacteria out of 60 isolates were root growth promoters (Narula et al., 2013b).

Phosphate solubilization by endophytes

Another important beneficial attribute of endophytic bacteria is P solubilization. The improvement of soil fertility is one of the most common strategies to increase agricultural production. The biological nitrogen fixation is very important in enhancing the soil fertility. In addition to biological nitrogen fixation, phosphate solubilization is equally important. Phosphorus (P) is one of the major essential macronutrient for biological growth and development. Microorganisms offer a biological rescue system capable of solubilizing the insoluble inorganic P of soil and make it available to the plants. The ability of some microorganisms to convert insoluble P to an accessible form, like orthophosphate, is an important trait in a plant growth promoting bacteria for increasing plant yields (Rodriguez et al., 2006).

The use of phosphate solubilizing bacteria as inoculants increases the P uptake by plants (Chen et al., 2006). Phosphate solubilizing bacteria secrete organic acids and phosphatases to solubilize insoluble phosphate to soluble form (Kim et al., 1998). The most efficient phosphate solubilizers belong to genera *Bacillus, Rhizobium* and *Pseudomonas* amongst bacteria, and *Aspergillus* and *Penicillium* amongst fungi. A total of 98 non-symbiotic endophytic bacterial strains were isolated from soybean root nodules grown in Heilong Jiang province of China and most of the strains could solubilize mineral phosphate (Li et al., 2008). Matsuoka et al. (2013) isolated endophytic bacteria (e.g. *Bacillus* sp., *Streptomyces luteogriseus* and *Pseudomonas fluorescens*) from *Carex kobomugi* roots, which exhibited both inorganic phosphate solubilization and siderophore production under Fe or P limiting conditions. Their results suggested that colonization of root tissue by these bacteria contribute to the Fe and P uptakes by *C.*

kobomugi by increasing availability in the soil.

Another study showed that a total of 38.3% out of 60 nodule endophytic bacteria from fieldpea were phosphate solublizers and isolate PNE15 was the best phosphate solubilizer among all isolates (Narula et al., 2013a). In cases of chickpea, 12 endophytic bacteria from roots and 76 from the nodules were also screened for P solubilization. Results showed that 41.7% of isolates from roots and 73.6% from nodules were solubilizing phosphate and CRE3, and CNE215, were highest P solublizers (Saini et al., 2013b). Further, 136 nodule and 90 root endophytic bacterial isolates were obtained from roots and nodules of legumes and non-legumes. In legume roots, 47.8% and in nodules 56% of bacterial endophytes were solubilizing P (Kumar et al., 2013).

Metabolite production by endophytes

Various types of secondary metabolites like antibiotics, organic acids, ammonia, enzymes and growth hormones (type of organic acids) are produced by the bacterial endophytes. These metabolites beneficially affect the plant directly or indirectly. Ammonia fulfills the demand of nitrogen of plants and organic acids helps in solubilization of insoluble nutrients. A number of different scientists have reported metabolite production by endophytes. Hung et al. (2007) reported that out of 109 bacterial endophytes, 33% were producing pectinase enzyme and 51% of endophytes were producing cellulase enzyme from soyabean. Similarly, out of 91 bacterial isolates from roots of coastal sand dune plants, 23 produced protease, 37 produced pectinase, and 38 produced chitinase (Dong-Sung et al., 2007). Organic acid producing endophytic bacterial strains have been isolated by Forchetti et al. (2007) from roots of sunflower. Three strains that were grown in control medium produced jasmonic acid (JA), 12-oxo-phytodienoic acid (OPDA) and abscisic acid (ABA). These three strains did not differ in amount of JA or OPDA produced, however ABA content was higher than that of JA, and production of both ABA and JA increased under drought condition. Li et al. (2013) isolated eleven bioactive alkaloids produced from *Pseudomonas brassicacearum* subsp. *neoaurantiaca*, an endophytic bacterium from *Salvia miltiorrhiza*. All these compounds were isolated from this bacterium for the first time. The antifungal and antibacterial activities of these compounds were evaluated. The results indicate that some cyclodipeptides may play an important role in plant-bacteria interactions.

Narula et al. (2013a), found that out of a total of 60 field pea nodule endophytic bacteria, 83.3% were ammonia producers and isolate PNE15 was the best ammonia producer and 32% were organic acid producers and isolate PNE17 was found to be the best organic acid producer. Saini et al. (2013b), isolated 88 endophytic bacteria from roots and nodules of chickpea. Large number of bacterial endophytes from roots (75%) and nodules (80.3%) were ammonia producing with CRE 12 and CNE76 being highest ammonia producer.

Siderophore production by endophytic bacteria

Bacterial siderophores are low molecular weight compounds with high iron (III) chelating affinity (Sharma and Johri, 2003) that are responsible for the solubilization and transport of iron (III) into bacterial cells. Iron is an essential mineral and its sequestration by specific bacterial siderophores may induce the development of plant disease (Nachin et al., 2003). Acquisition of iron from siderophores produced by other microbial species has been described for *Escherichia coli*, *Salmonella typhimurium* (Martinez et al., 1990) and *Actinobacillus pleuropneumoniae* (Diarra et al., 1996).

Paulo et al. (2006) evaluated the ability of *Methylobacterium* sp., isolated as citrus endophytic bacteria to produce siderophores. All strains of *Methylobacterium* sp. tested were CAS-positive for siderophores production and the siderophores production tested by the CAS-agar assay revealed that 66% of CVC-symptomatic, 55% of uninfected, 20% of asymptomatic and 10% of tangerine strains of *Methylobacterium* sp., showed very high production.

Catherine et al. (2012) isolated 43 bacterial endophytes and assessed siderophore production. Distinct orange halos were observed with all the 12 *Pseudomonas* isolates with *Flavimonas oryzihabitans* isolates having the largest orange halos. They suggested that *Pseudomonas* isolates could therefore be considered high siderophore producers.

Biocontrol activities of endophytic bacteria

Endophytes play an important role in protection of host plants from infection by phytopathogens. Endophytes are not subjected to the competition from soil microbes and colonize in the plant tissue. They have the ability to penetrate plant cells, stimulate plant defense response and produce antifungal metabolites *in situ*. A large number of endophytic microbes have been studied for their potential role as biocontrol agents against *Fusarium* (Lixiang et al., 2004). *Fusarium* wilt is a fungal disease, which affects a broad range of plants. The biological approach to control *Fusarium oxysporum* is becoming popular in many crop plants. Edkona et al. (2013) isolated five endophytic bacteria, exhibiting potential to control *F. oxysporum* from black pepper roots.

Ma et al. (2013) isolated endophytic bacteria from five different parts (root, stem, petiole, leaf and seed) of *Panax notoginseng* and evaluated antagonistic activity

against *F. oxysporum*, *Ralstonia* sp. and *Meloidogyne hapla*, three major pathogens associated with root-rot disease complex of *P. notoginseng*. Out of the 104 antagonists, 51 strains showed antagonistic activities to one pathogen only, while 43 and 10 displayed activities towards two and all three pathogens, respectively. The most dominant species in all tissues were *Bacillus amyloliquefaciens* subsp. *plantarum* and *Bacillus methylotrophicus*, which were represented by eight strains with broad antagonistic spectrum to all three test pathogens of root-rot disease complex of *P. notoginseng*. Similarly, 11 endophytic bacterial isolates from roots and nodules, and roots of non legumes also produced siderophores but showed low biocontrol activity against plant pathogens (Giri and Dudeja, 2014).

COLONIZATION AND HOST SPECIFICITY OF ENDOPHYTES

Recent studies show that endophytes are not host specific (Cohen, 2006). Single endophytes can invade a wide host range. Carroll and Petrini (1983) suggested that some strains of the same fungus isolated from different parts of the same host differ in their ability to utilize different substances. So endophytes can be isolated from different plants belonging to the different families and classes and grow under different ecological and geographical conditions. Host endophyte relationship may be variable from host to host and endophyte. Some research showed that host plant and endophyte relationship are able to balance pathogen-host antagonism and are not truly symbiotic one (Schulzt et al., 1999).

Criteria to recognize true endophytic bacteria require not only the isolation from surface disinfected tissues but also microscopic evidence to visualize bacteria inside plant tissues (Sagarika et al., 2010). Naturally occurring endophytes can be visualized by fluorescence *in situ* hybridization (FISH) combined with confocal laser scanning microscopy using specific probes, as well as scanning electron microscopy (SEM), transmission electron microscopy (TEM) and phase contrast microscopy (Amann et al., 1990; Loy et al., 2007). SEM studies confirmed abundant bacterial colonization of the proximal parts of wheat root surface. Nautiyal (2000) has reviewed other developments including use of different markers in the study of root colonization. Microscopic studies of *gfp* tagged endophytic inoculants revealed highly heterogeneous colonization patterns.

Colonization of bacteria in rhizosphere or on plant surface is a complex process which involves interplay between several bacterial traits and genes. The colonization is multistep process and includes: (a) migration towards root surface, (b) attachment, (c) distribution along root and (d) growth and survival of the population. For endophytic bacteria, one additional step is required, that is, entry into root and formation of microcolonies inter or intra-cellularly. Each trait may vary for different associative/endophytic bacteria. Colonization of bacteria is traced by tagging the putative colonizing bacteria with a molecular marker such as auto fluorescent marker (*gfp*) or β-glucosidase (*gus*) followed by electron or confocal laser scanning microscopy (Reinhold-Hurek and Hurek, 2011). Fluorescent *in situ* hybridization with real time PCR analysis can also be used for tracking bacterial colonization and its quantification (Lacava et al., 2006). Understanding of molecular mechanism involved in associative or endophytic colonization process is not well understood. Recent reports based on the genomic data and other similar reports have suggested resemblance of colonization methods between pathogenic bacteria and PGPB (Hardoim et al., 2008).

Recognition/chemotaxis

The sequence of events leading to colonization of a plant by a bacterium that is to become endophytic is presumably similar, at least in the early stages, to that observed for rhizoplane or rhizosphere bacteria. Bacteria belonging to the ‘root colonizing rhizosphere competent bacteria’ e.g. *P. fluorescens*, *Azospirillum brasilense* and *Bacillus subtilis*, all common rhizosphere inhabitant,s are often found as colonizers of the internal tissue of plants (Hallmann and Berg, 2006). Lugtenberg and Dekkers (1999) found that bacterial colonization of roots often starts with the recognition by bacteria of specific compounds that are secreted by the root tissue. De Weert et al. (2002) observed tomato roots secreting organic as well as amino acids in their exudates which provide chemo-attractants for *P. fluorescens* strain WCS365, but sugars had no effect on the chemotactic response.

Many biotic and abiotic factors affect root exudation. Spatial and temporal exudation patterns have been observed along the axes of the roots, creating differential niches for diverse soil bacteria (Kuzyakov, 2002). Hence, one might hypothesize that different root zones (the cork zone, root hair, elongation zone, differentiation zone and root cap) create a range of spatial niches that select specific bacterial communities, allowing establishment of interactions with the plant. For instance, colonization of wheat roots by *A. brasilense* strain 245 occurs preferentially at the root hair zone and at the sites of lateral root emergence (Broek et al., 1999) while colonization of rice roots by *Azoarcus* sp. strain BH72 occurs preferentially in the zones of division and elongation just behind the root cap (Hurek et al., 1994) or for rhizobial species at those of lateral root emergence (Chi et al., 2005).

In a study, 11 most efficient isolates were selected out

of more than 200 endophytic bacteria isolated previously from roots of chickpea (*Cicer arietinum*), field pea (*Pisum sativum*), lucerne (*Medicago sativa*), wheat (*Triticum aestivum*) and oat (*Avena sativa*) and nodules of chickpea and field pea (Giri and Dudeja, 2013a). To know their extent of establishment in different host and non-host tissues root exudates of four hosts' chickpea, field pea, wheat and oat were collected at 7 and 14 days growth. Root exudates of chickpea, field pea, wheat and oat attracted different endophytic bacteria to different extent. Isolate ORE27 exhibited maximum chemotactic ratio towards root exudates of all crops followed by isolate WRE4 towards oat, wheat and chickpea root exudates. Presence of root exudates, promoted the growth of different endophytes in MS medium tubes. In field pea roots, maximum endophytic colonization after 21 days was observed followed by chickpea, oat and wheat. All the 11 endophytic bacteria entered pea roots, 10 in chickpea, eight in oat and wheat roots in MS medium tubes. No host specificity among 11 endophytic bacteria and four hosts could be observed at any of the stage of root colonization.

Root colonization

Root colonization is the first and the critical step in establishment of plant-microbe association. Microorganisms move towards rhizosphere in response to root exudates, which are rich in amino acids, organic acids, sugars, vitamins, purines/pyrimidines and other metabolic products. In addition to providing nutritional substances, plants start cross-talk to microorganisms by secreting some signals which cause colonization by some bacteria while inhibiting the other (Bais et al., 2006; Compant et al., 2011). Root hair regions and emergence points are preferred site for colonization (Lugtenberg and Kamilova, 2009). Colonization of roots by microorganisms may further induce release of exudates, and create 'biased' rhizosphere by exudating specific metabolic products. In some rhizospheric bacteria, root exudates induce flagellar motility that leads their colonization on plant surfaces. Lugtenberg and Kamilova (2009) demostrated the role of bacterial major outer membrane protein (MOMP) in early host recognition where MOMPs from *Azospirillum brasilense* showed stronger adhesion to extracts of cereals than extracts of legumes and tomatoes. It suggests involvement of MOMPs in adhesion, root adsorption and cell aggregation of the bacterium. Preston et al. (2001), identified SSIII secretion system in *P. fluorescens* SBW25 by *in vitro* expression technology (IVET), a promoter trapping technique.

Using 11 most efficient isolates selected out of more than 200 endophytic bacteria isolated previously from roots of chickpea (*Cicer arietinum*), field pea (*Pisum sativum*), lucerne (*Medicago sativa*), wheat (*Triticum aestivum*) and oat (*Avena sativa*) and nodules of chickpea and field pea, root colonization studies were done (Giri and Dudeja, 2013b). Extent of establishment in different host and non-host tissues four host chickpea, field pea, wheat and oat with these bacteria in sterilized sand in disposable coffee cups was studied. To induce nodulation in chickpea and field pea were also inoculated with respective rhizobia, apart from different endophytes. In chickpea at 15 days, isolate ORE 27, at 30 days, isolates CNE 215 and ORE 35 and at 50 days in total, 6 bacteria were able to enter the roots and exit as endophytic. In the case of field pea all the 11 bacteria entered the roots after 30 days of growth, whereas in wheat and oat bacteria entrance was detected at 50 days and not at 15 or 30 days. In wheat 4 and in oat 6 bacterial isolates were detected as endophytic. Even at 50 days, neither in chickpea nor in field pea nodules, no bacterial isolate was able to enter. In chickpea roots maximum endophytic colonization was observed by isolate PNE 92, in field pea by isolates CNE1 and PNE 17; in wheat and oat, isolate ORE 27 showed highest root endophytic colonization. No host specificity among endophytic bacteria and different hosts could be observed.

Entry in plant tissue as endophyte

Entry of endophytic bacteria in plant roots is known to occur (a) through wounds particularly where lateral or adventitious roots occur; (b) through root hairs and (c) between undamaged epidermal cells (Hardoim et al., 2008). Chi et al. (2005) demonstrated that the colonization of *gfp* tagged rhizobia in crop plants begin with surface colonization of the rhizoplane at lateral root emergence, followed by endophytic colonization within roots, and then ascending endophytic migration into the stem base, leaf sheath, and leaves where they develop high populations.

Preito et al. (2011) found that *Azospirillum* may also colonize endophytically through wounds and cracks of the plant root. Endophytic bacteria may colonize root tissues and spread actively in aerial parts of plants through expressing moderate amount of degradative enzymes such as pectinases and cellulases. Utilization of aforesaid enzymatic activities for colonization by *Azospirillum irakense*, *Azoarcus* sp. and others has been demonstrated as one of the efficient methods to get entry into the host plant. Endoglucanase is one of the major determinants for the colonization of endo rhizosphere, which was evident from the observation that *Azoarcus* strain lacking endoglucanse was not effective in colonizing the rice plants. The endoglucanase loosen larger cellulose fibers, may help in entry to the plant. A homologue of endoglucanase gene has also been identified in *P. stutzeri* A1501, which occasionally colonizes cortex of crop plants. In addition to endogluca-

canase, exoglucanases may also help in colonization process. Reinhold-Hurek and Hurek (2011) identified an exoglucanase having cellobiohydrolase and β-glucosidase activity, playing a key role in colonization process of *Azoarcus* sp. BH72. Endophytic colonization is not as specific as of rhizobia but successful endophytic colonization does involve a compatible host plant (Ryan et al., 2008). However, endophytic colonization indeed depends upon the physiological changes in plants and is restricted or slowed down by defense mechanism (Rosenblueth and Martínez-Romero, 2006). Understanding of molecular mechanism and conditions limiting the colonization process need to be elucidated for exploiting the beneficial endophytic or associative interaction with plants.

BENEFICIAL EFFECTS OF ENDOPHYTIC BACTERIA AS INOCULANTS

Plant growth promotion has been shown for many endophytic bacteria (Zachow et al., 2010; Malfanova et al., 2013). Direct plant growth promotion mediated by endophytes is mostly based on providing essential nutrients to plants and production and/or regulation of phytohormones. Large number of endophytes has been reported in various plant tissues and these endophytes possess different beneficial properties. Bacterization of these endophytes promotes plant growth and in case of legumes helps in fixation of more nitrogen and is important aspect for the success of these endophytes in agriculture ecosystems. However, only a few studies using endophytes as inoculants under pot culture conditions are available. No report under field conditions could be observed. Such studies therefore need more attention.

Bacterization of bacterial endophytes promoted growth of red clover more often when applied in combination with *R. leguminosarum* biovar *trifolii* than when applied singly (Sturz et al., 1997). However, *Bacillus megaterium*, *Bordetellla avium* and *Curtobacterium luteum* consistently promoted growth either individually or in combination with *R. leguminosarum* biovar *trifolii.* Nodulation was promoted when *R. leguminosarum* biovar *trifolii* was coinoculated with *Bacillus insolitus, B. brevis* or *Agrobacterium rhizogenes*.

A total of 166 endophytic bacteria isolated from root of chickpea (*Cicer arietinum*), pea (*Pisum sativum*) and lucerne (*Medicago sativa*), and non-legumes wheat (*Triticum aestivum*) and oat (*Avena sativa*) and nodules of chickpea were found to promote the growth of chickpea roots in chickpea root growth promotion assay in agar plates (Saini et al., 2013b), however chickpea nodule endophytic bacteria were better root growth promoters as compared to others. Selected 79 endophytic bacterial isolates were inoculated together with *Mesorhizobium* in chickpea under pot culture conditions and showed enhanced plant growth, nodulation and nitrogen fixing parameters in chickpea, particularly, endophytic bacterial isolates in combination with *Mesorhizobium* than *Mesorhizobium* alone. Plant growth promoting endophytic isolates isolated from nodules of chickpea showed the highest growth promotion and enhanced nitrogen fixation in terms of shoot dry weight and shoot N contents. The most efficient isolates CNE1036 was identified as *Bacillus subtilis* and isolate LRE 3 was identified as *Bacillus amyloliquefaciens* by sequencing of amplified 16S rDNA. Similarly, bacterial endophytes isolated from field pea were inoculated along with standard *R. leguminosarum* biovar *trifolii* strain PS-43 and some of the co-inoculations showed enhanced nodulation, root growth, plant growth and nitrogen content in shoot of fieldpea (Narula et al., 2013a).

Two strains of *Paenibacillus macerans* promoted plant growth of *Cymbidium eburneum* orchid under greenhouse conditions (Faria et al., 2013). None of the treatments had a deleterious effect on growth of inoculated plants. Their results suggested that these bacterial effects could be potentially useful to promote plant growth during seedling acclimatization in orchid species other than the species of origin. Due to the presence of multiple traits in endophytic bacteria, enhancement in crop productivity in all the tested crops was observed.

CONCLUSION

The root system, which was traditionally thought to provide anchorage and uptake of nutrients and water, is a chemical factory that mediates numerous underground interactions. Plants release enormous amount of chemicals through their roots, at a significant carbon cost, to combat pathogenic microorganisms and attract beneficial ones. Roots of legume and non-legume plants normally associate with diverse microorganisms. Some of bacteria and fungi are capable of entering the plant roots as endophytes and establishes a mutualistic association. Many reports found in literature strongly suggest that endophytes have an excellent potential to be used as plant growth promoters with legumes and non-legumes. Plants constitute vast and diverse niches for endophytic organisms. The processes of host-microbe signaling and colonization, and the mechanisms leading to mutual benefits are less well characterized. It seems no host specificity between host and endophytic bacterial population exist. However interaction studies at molecular level are required to exactly pin point the existence of host specificity and quantum of benefits derived by the host.

Conflict of Interests

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

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Vol. 8(15), pp. 1573-1579, 9 April, 2014
DOI: 10.5897/AJMR2013.6138
Article Number: 6B51EC143818
ISSN 1996-0808
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http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Screening and evaluation of *Lactobacillus* spp. for the development of potential probiotics

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Received 1 July, 2013; Accepted 17 March, 2014

Probiotics are live microbial food ingredients that have beneficial effects on consumption. The present study searched fermented food samples for potential lactobacilli that could be used for the development of novel gastrointestinal probiotics. Fifty five (55) isolates were obtained including 46 bacterial and 9 yeast isolates. On the basis of morphological and biochemical properties, 21 were identified as *Lactobacillus* spp. Systematic procedures were used to evaluate the probiotic properties of the *Lactobacillus* isolates including carbohydrate fermentation, autoaggregation, acid tolerance, bile resistance and pancreatin tolerance test. 85% of the examined *Lactobacillus* spp. showed the ability to form auto-aggregate, 75% can withstand bile while all the strains were able to tolerate pancreatin. Commercially available probiotic strain *Lactobacillus casei* was isolated and tested for functional criteria required to be a beneficial probiotics and results were compared.

Key words: *Lactobacillus*, probiotics, fermented food, auto-aggregation, bile tolerance.

INTRODUCTION

The concept of probiotics evolved at the turn of 20th century from a hypothesis first proposed by Nobel Prize winning Russian scientist Elie Metchnikoff (Bibel, 1988), who suggested that the long, healthy life of Bulgarian peasants resulted from their consumption of fermented milk products because the fermenting bacilli (*Lactobacillus*) positively influenced the microflora of the colon, decreasing toxic microbial activities (Sanders and Huisin't, 1999). Probiotics can be classified as "live microorganisms that, when consumed in an adequate amount, confer health benefit on the host" (Join FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Foods. London, Ontario, April 30 and May 1, 2002:1-11). The major bacterial species that have been considered as probiotics over the years are *Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium longum* and some other *Bifidobacterium* species (Arvanitoyannis and Houwelingen-Koukaliaroglou, 2005).

The potential and established health benefits associated with the usage of probiotics are: prevention of enterocolitis, sepsis (in very low birth weight infants),

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Abbreviations: IBS, Irritable bowel syndrome; **MRS,** Man Rogosa Sharpe; **OHOL,** obligately homofermentative lactobacilli; **FHEL,** facultatively heterofermentative lactobacilli; **OHEL,** obligately heterofermentative lactobacilli; **µL,** micro litre; **MR-VP,** methyl red VogesProskauer; **WHO/FAO,** World Health Organization/Food and Agriculture Organization; **w/v,** weight/volume.

diarrhea and *Helicobacter pylori* infections and prevention of cancer, heart disease, irritable bowel syndrome (IBS), ulcerative colitis (in adults), rheumatoid arthritis, nasal and food allergies, atopic dermatitis by competitive exclusion of pathogens, stimulation of mucus secretion and modulation of immune response (Leroy et al., 2008).

The main factors that promote growth in the global market of probiotics are increasing levels of health-consciousness and the availability of probiotics in the form of dietary supplements. The market of probiotic products generated US$15.9 billion in 2008 and is forecasted to reach US$ 28.8 billion in 2015. The major factors that have facilitated market growth of probiotics are the appropriate components for formulation and the scientific knowledge of the provided benefits (Markets and Markets, 2009; Global Industry Analystic, 2010).

Probiotic microorganisms are host specific; thus a strain selected as a probiotic for one animal may not be suitable in another animal. Furthermore, microorganisms selected for probiotic use should exhibit certain characteristics, they must be able to adhere to intestinal mucosa, easily cultivable, should be non-toxic, non-pathogenic to the host and moreover exert beneficial effect on the host. The microorganisms must be able to produce useful enzymes or physiological end products that can be used by the host. They should remain viable for long time and withstand acidic pH of the stomach (Leroy et al., 2008). Hence the current investigation was planned to isolate best suited microorganisms from the fermented food stuffs.

MATERIALS AND METHODS

Isolation of lactobacilli strains

Twenty seven fermented food samples like home made curd, dosa batter, idli batter, jalebi batter, lassi and yogurt were collected in pre-sterilized containers aseptically and stored at 4°C till further processing. One gram of appropriately diluted sample was spreaded on freshly prepared Man RogosaSharpe (MRS) agar and incubated at 30°C for 24-72 h. Isolated colonies of distinct morphology were sub-cultured on fresh MRS plates. All the isolates were preserved as glycerol stocks at -20°C for further use.

Phenotypic/physiological identification of isolates

Provisional identification of *Lactobacillus* spp. was based on their ability to grow in MRS broth, positive Gram reaction, absence of endospore and rod shaped cells. Biochemical characterization of the isolates was carried out by evaluating the production of catalase, oxidase, arginine hydrolysis, methyl red Voges Proskauer (MR-VP) reactions, nitrate reduction test, and starch hydrolysis test (Hammes and Hertel, 2009). The *Lactobacillus* was categorized on the basis of fermentation group and physiological properties of isolates. The fermentation of glucose without gas, growth at 37°C and no growth at 15°C identifies obligately homofermentative lactobacilli (OHOL); growth both at 15 and 37°C without gas production is characteristic of facultatively heterofermentative lactobacilli (FHEL), whereas gas production at 37°C and variable growth at 15°C are characteristic of obligately heterofermentative lactobacilli (OHEL) (Koll et al., 2010).

Testing of auto-aggregation ability

Auto aggregation ability is the indicator of adhesion ability and tolerance of gastrointestinal environmental conditions, which is considered as prerequisite for screening lactobacilli for their functional properties. Lactobacilli were grown for 48 h at 37°C on MRS agar plates. 10 µL of culture was suspended on a glass microscope slide in 1 ml of 0.9% saline solution (pH 6.7). Auto-aggregation was then determined by the ability to form aggregates (clearly visible sand-like particles) within 2 min at room temperature. The results were expressed as: score 0- no auto-aggregation, score 1- intermediate autoaggregation (presence of some flakes), and score 2- strong auto-aggregation (Pascual et al., 2008).

Testing of acid tolerance

The effect of low pH on the survival of lactobacilli was examined by inoculating 1% of freshly prepared seed culture in 20 mL MRS broth with pH 2.0, 5.0 and 7.0 followed by incubation at 37°C and 200 rpm for 24-48 h. Growth was measured by taking OD after a time interval of 24 and 48 h at 620 nm (Systronics, Japan). Increase in optical density was considered as reflection of growth at 37°C (Koll et al., 2008).

Testing of bile tolerance

The effect of bile on the survival of lactobacilli was examined by inoculating 1% 24 h old culture in MRS broth containing oxbile (2% w/v) followed by incubation at 37°C and 200 rpm (Koll et al., 2010). Growth was measured by taking OD after a time interval of 24 and 48 h at 620 nm (Systronics, Japan). Increase in optical density was considered as reflection of growth at 37°C.

Testing of pancreatin tolerance

The effect of pancreatin on the survival of lactobacilli was examined by inoculating 1% 24 h old culture in MRS broth containing pancreatin (0.5% w/v) followed by incubation at 37°C and 200 rpm for 24-48 h (Koll et al., 2010). Growth was measured by taking OD after a time interval of 24 and 48 h at 620 nm (Systronics, Japan). Increase in optical density was considered as reflection of growth at 37°C.

RESULTS AND DISCUSSION

Isolation of lactobacilli strains

Fifty five (55) strains were isolated from 27 fermented food items. Of these, 46 were bacterial isolates and nine were yeast isolates. Based on colony morphology, 55 isolates were selected for further study. The purified colonies of isolates were stored on slants and glycerol stocks at 4 and -20°C, respectively. Fermented food samples are considered as good source of *Lactobacillus* spp. The samples used for the study are indigenous part of diet of the Indian people. Fermented foods including fermented dairy products are known to have beneficial health effects in addition to improving food safety. Many

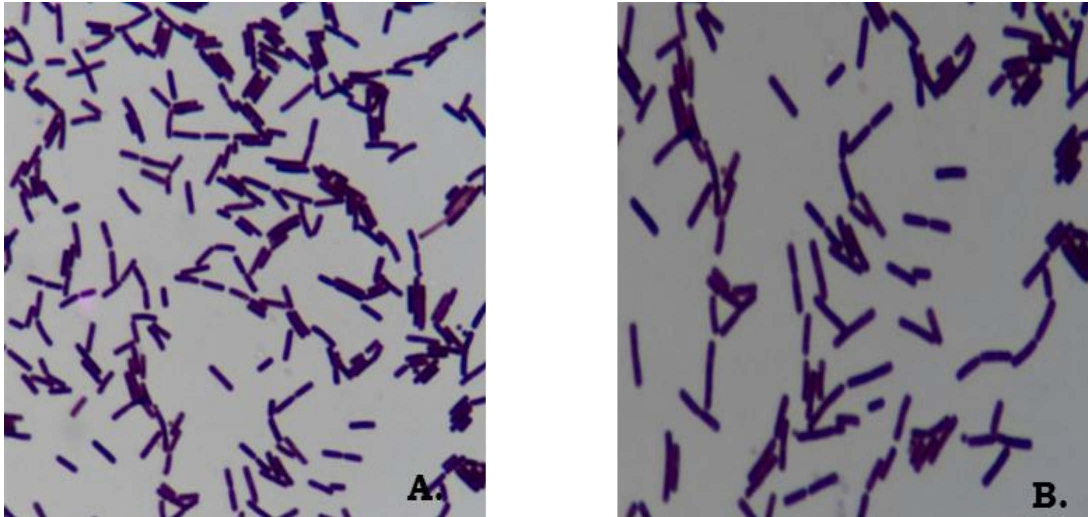

Figure 1. Morphological features of isolates under light microscope after Gram's reaction (100x) A. NKC17(3B); B. NKC18(5C).

Lactobacillus spp. have been used for the fermentation and as natural means of preserving perishable foods. Erdogrul et al. (2006) isolated 21 strains from ten fermented food samples. *Lactobacillus* spp. has a long history of natural occurrencein a variety of food products and has GRAS status. Clinical studies underline the safe use of *Lactobacillus* in humans (Todorov and Dicks, 2008).

Morphological and biochemical identification of isolates

The colonies with pure white glistening, convex, small (2-3mm diameter) with regular margins were subcultured on MRS agar to obtain pure culture. The provisional identification of lactobacilli was based on the growth of Positive Gram's reaction, non endospore forming rods on MRS agar with negative catalase reaction (Hammes and Hertel, 2009). Out of 46 bacterial isolates, 40 were found to be Gram positive, 26 isolates were without endospore and among them 21 gave negative catalase reaction (Figure 1) and hence considered as lactobacilli.

Catalase and oxidase tests indicated the negative nature of the isolates (MacFaddin, 1980). All twenty-one isolates including the control strain (*Lactobacillus casei*) isolated from yakult were found to be oxidase and catalase negative. Similar findings were recorded by Erdogrul et al. (2006). Arginine hydrolysis was performed in MRS broth containing 0.3% arginine (Spano et al., 2002). In the current study, sixteen strains out of twenty-one were able to hydrolyze arginine. *Lactobacillus* vary in their ability to degrade arginine and those able to derive energy from arginine catabolism may be more competitive in the stressful environment of wine (presence of acid and alcohol) than those stains that are unable to degrade arginine (Liu and Pilone, 1995; Spano et al., 2002).

All the 21 isolates were found to be MR-VP negative, and were unable to hydrolyze starch and reduce nitrate. Hammes and Hertel (2009) have reported that *Lactobacillus* spp. are unable to reduce nitrate and hydrolyze starch. Findings of the current study are comparable to that of Forouhandeh et al. (2010) in which they reported that none of their isolates exhibited nitrate reduction and starch hydrolysis.

For detecting fermentation group, some physiological properties were assessed using glucose as the test sugar. In the present study out of 21 isolates, eight were identified to be OHOL; seven were identified to be FHEL; six were identified to be OHEL (Table 1).

Testing of auto-aggregation ability

Aggregation is important for biofilm formation and may assist probiotic bacteria to adhere to mucus and epithelial cells and survive harsh conditions in the gastrointestinal tract (Lepargneur and Rousseau, 2002; Reid and Burton, 2002). Adhesion ability is considered as a prerequisite for screening intestinal lactobacilli for their functional properties (Collado et al., 2007; Mercenier et al., 2008; Sathyabama et al., 2012). In the current study, three strains showed no auto-aggregation ability, nine strains showed intermediate auto-aggregation ability and nine strains including NKY1H1 (commercially available probiotic strain) showed strong auto-aggregation ability (Table 2). Koll et al. (2010) have reported that 59% of their test strains showed auto-aggregation and 30 strains were strongly autoaggregative. Auto-aggregationis strain-

Table 1. The *Lactobacillus* strains showing acid and gas production from glucose-MRS broth at 15 and 37°C.

Strain ID	At 15°C (48 h)		At 37°C (48 h)	
	Acid	Gas	Acid	Gas
NKI1A1	+	-	+	-
NKC1F2	+	+	+	+
NKY1H1	-	-	+	-
NKC5J2	-	-	+	-
NKJ1K1(L)	+	+	+	+
NKC6L2	+	-	+	-
NKC7M1	+	-	+	-
NKC9O3	+	+	+	+
NKJ1K2(S)	+	+	+	+
NKC11R1	-	-	+	-
NKC12S2	-	-	+	-
NKC13T2	-	-	+	-
NKD2V1	+	-	+	-
NKC17(1B)	+	+	+	+
NKL1(2B)	-	-	+	-
NKL1(2C)	+	-	+	-
NKC17(3A)	+	-	+	-
NKC17(3B)	-	-	+	-
NKC18(4A)	+	-	+	-
NKC18(5B)	+	+	+	+
NKC18(5C)	-	-	+	-

NKY1H1 commercially available probotic strain (*Lactobacillus casei*); OHOL obligately homofermentative lactobacilli (- - + -); FHEL facultatively heterofermentative lactobacilli (+ - + -); OHEL obligately heterofermentative lactobacilli (+ + + +).

Table 2. Auto-aggregation ability of isolates.

Strain ID	Auto-aggregation score
NKI1A1	1
NKC1F2	1
NKY1H1	2
NKC5J2	0
NKJ1K1(L)	1
NKC6L2	2
NKC7M1	1
NKC9O3	2
NKJ1K2(S)	2
NKC11R1	0
NKC12S2	1
NKC13T2	1
NKD2V1	2
NKC17(1B)	1
NKL1(2B)	2
NKL1(2C)	0
NKC17(3A)	1
NKC17(3B)	2
NKC18(4A)	1
NKC18(5B)	2
NKC18(5C)	2

NKY1H1 commercially available probotic strain (*Lactobacillus casei*); Score 0- no auto-aggregation; score 1- intermediate auto-aggregation; score 2- strong auto-aggregation.

specific and most probably involves species-specific surface proteins (Todorov and Dicks, 2008).

Testing of acid tolerance

Probiotic strains have to survive harsh conditions in the gastrointestinal tract (GIT), e.g. pH values ranging from 1.0 to 3.0 in the stomach (Mainville et al., 2005). Resistance to low pH is thus important criteria for the selection of probiotic bacteria (Havenaar et al., 1992).The effect of low pH was studied to determine the ability of twenty-one isolates to survive at low pH of stomach during their passage through gastrointestinal tract (Koll et al., 2010). In this study, at pH 2 isolate NKC12S2 showed good growth after 48 h of incubation. At pH 5, isolates NKC5J2, NKC17(3B) andNKL1(2B) showed good growth after both 24 h and even after 48 h of incubation. NKC17(1B) showed good growth after 24 and 48 h of incubation. NKC17(3A) and NKC18(5C) showed good growth after 24 h incubation but growth declined after 48 h of incubation. Strain NKY1H1 showed fair growth at both pH after 24 h of incubation but this growth declined significantly after 48 h of incubation (Table 3).

In some reported cases, none of the strains grew at pH 2.5 and only very few were reasonably acid tolerant (Jacobsen et al., 1999; Awasthy et al., 2008). This difference in acid tolerance of strains from two species within the same genus may be due to differences in the cell wall structure (Conway et al., 1987).

Testing of bile tolerance

Before reaching the distal part of the intestinal tract and exerting their probiotic effect, these bacteria must survive during transition through the stomach and upper part of the intestinal tract (Bao et al., 2010). Resistance to elevated concentration of bile salt is an important selection for probiotic bacteria in order to survive harsh environment of gastrointestinal tract (Todorov and Dicks, 2008). Lan-Szu and Bart (1999) have substantiated that strains selected as probiotic bacteria should tolerate bile for at least 90 min, which is the time needed to cross the barrier; in the present study, tolerance was checked for 24 and 48 h of incubation. Although the bile concentration of human gastrointestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% w/v (Sjovall, 1959; Gilliland et al., 1984). In the present study, bile

Table 3. Effect of pH on the growth of selected isolates (OD at 620 nm).

Strain ID	pH 2		pH 5	
	O. D. After 24 h	O. D. After 48 h	O. D. After 24 h	O. D. After 48 h
NKI1A1	0.490	0.150	0.896	0.671
NKC1F2	0.465	0.440	0.788	0.650
NKY1H1	0.422	0.115	0.731	0.688
NKC5J2	0.118	0.118	1.052	1.209
NKJ1K1(L)	0.437	0.120	0.744	0.810
NKC6L2	0.336	0.635	0.842	0.680
NKC7M1	0.203	0.712	0.812	0.679
NKC9O3	0.174	0.600	0.726	1.312
NKJ1K2(S)	0.379	0.461	0.767	0.701
NKC11R1	0.120	0.117	0.733	0.676
NKC12S2	0.572	1.030	0.729	0.665
NKC13T2	0.796	0.668	0.619	0.634
NKD2V1	0.589	0.159	0.679	0.712
NKC17(1B)	0.472	0.704	0.770	1.564
NKL1(2B)	0.628	0.408	1.465	1.404
NKL1(2C)	0.153	0.136	0.754	0.651
NKC17(3A)	0.959	0.128	1.061	0.750
NKC17(3B)	0.753	0.135	0.957	1.801
NKC18(4A)	0.118	0.393	0.774	0.626
NKC18(5B)	0.538	0.143	0.713	0.921
NKC18(5C)	0.858	0.157	2.040	0.737

NKY1H1 commercially available probotic strain (*Lactobacillus casei*).

Table 4. Tolerance of the selected strains towards bile and pancreatin (OD at 620 nm).

Strain ID	Bile		Pancreatin	
	O. D. after 24 h	O.D. after 48 h	O. D. after 24 h	O.D. after 48 h
NKY1H1	0.325	0.263	0.356	0.811
NKC6L2	0.421	0.221	0.548	6.020
NKC9O3	0.334	0.730	0.835	7.347
NKJ1K2(S)	0.689	6.120	0.339	0.705
NKD2V1	0.786	8.13	0.228	0.683
NKL1(2B)	0.267	0.611	0.321	0.657
NKC17(3B)	0.498	0.668	0.483	5.610
NKC18(5C)	0.372	0.641	0.532	6.021

NKY1H1 commercially available probotic strain (*Lactobacillus casei*).

concentration of 2% w/v was taken. In this study, eight strains were tested for bile tolerance. Interestingly, six strains NKJ1K2(S), NKD2V1,NKC9O3, NKL1(2B), NKC17(3B) and NKC18(5C) not only survived high bile concentration but showed equally good growth after both 24 and 48 h. Commercially available probiotic strain NKY1H1 showed poor growth after incubation period of 24 h and the growth declined even more after 48 h incubation (Table 4).

This property may provide these strains with an advantage *in vivo* because upon exposure to bile acids, cellular homeostasis disruptions causes the dissociation of lipid bilayer and integral protein of their cell membranes, resulting in leakage of bacterial content and ultimately cell death. Resistance to ox-bile has been recorded for *Lactobacillus acidophilus* (Park et al., 2006), *Lactobacillus salivarius*241, *Lactobacillus plantarum*423 and *Lactobacillus curvatus*DF38 (Brink et al., 2006).

Testing of pancreatin tolerance

According to FAO/WHO (2006) and Mercenier et al. (2008) tolerance of gastrointestinal environmental conditions are considered as a prerequisite for screening intestinal lactobacilli for their functional properties. The effect of pancreatin was examined to determine the ability of eight isolates to survive during their passage through gastrointestinal tract (Koll et al., 2010). All tested strains were resistant to pancreatin at a concentration of 0.5% w/v and strain NKC9O3, NKC17(3B), NKC6L2 and NKC18(5C) showed excellent growth after 48 h incubation. Strain NKY1H1 showed average growth after 24 h incubation but the growth improved significantly after 48 h (Table 4). Koll et al. (2010) have reported resistance of all the test strains to pancreatin at 0.5% w/v.

Conclusion

This study was carried out for the isolation and identification of *Lactobacillus* species from traditional fermented foods that could be a means of ensuring safety. The probiotic properties of the isolates were comparable to commercially available *L. casei* (NKY1H1). In particular, strain NKL1(2B), NKC17(3B) and NKC18(5C) fulfilled the functional criteria required to be used as novel and beneficial probiotic. These three strains may be regarded as good candidate for *in vivo* studies and can be identified by specific molecular biology techniques.

Conflict of Interests

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

This research was supported by the Department of Microbiology, Chaudhary Charan Singh University, India. All the authors of the research paper and books cited are highly acknowledged.

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academicJournals

Vol. 8(15), pp. 1580-1585, 9 April, 2014
DOI: 10.5897/AJMR2013.6548
Article Number: 70A537043820
ISSN 1996-0808
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http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Prevalence of metallo-β-lactamases producers among carbapenem-resistant *Acinetobacter baumannii* strains isolated from diabetic foot ulcers

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Received 23 January, 2014; Accepted 17 March, 2014

The aim of this work was to detect the prevalence of metallo-β-lactamases (MBL) producers among carbapenem-resistant *Acinetobacter baumannii* strains isolated from diabetic foot ulcers. Out of 290 samples of diabetic foot ulcers, 99 strains of *A. baumannii* (33%) were isolated and identified by conventional culture methods. Antibiotic sensitivity pattern of the isolated *A. baumanni* strains was done by disc diffusion method. For *A. baumanni* strains that was resistant to imipenem and meropenem, MBL production was screened by potentiated disc test and confirmed by multiplex polymerase chain reaction for bla_{IMP} and bla_{VIM} genes. Out of 99 strains of *A. baumannii*, 26 (26%) were found resistant to imipenem and/or meropenem. Of these 26 carpabenem resistant *A. baumannii* strains 9 (34.61%) were positive for MBL by potentiated disc test, and 6 strains (23.07%) were positive for bla_{VIM} or bla_{IMP} by multiplex PCR; where bla_{VIM} gene was detected in 4 strains (15.38%) and bla_{IMP} was detected in 2 strains (7.69%). The antimicrobial susceptibility profile for the isolated *A. baumanni* strains showed that the highest sensitivity was to meropenem (74.4%), imepenem (76.55%), amikacin (65%) and the lowest sensitivity was to ceftazidime (11%) and ciprofloxacin (12%). Rapid dissemination of carbapenem-resistant isolates in diabetic foot ulcers is worrisome and calls for judicious use of antibiotics. bla_{VIM} and bla_{IMP} genes have a role in carbapenem-resistant in the community. More studies are needed to differentiate MBL from non-metalloenzymes producers.

Key words: Diabetic foot, *A. baumanni*, metallo-β-lactamases.

INTRODUCTION

Foot ulcers are among the leading causes of morbidity in diabetics and are the most common indication for admission in this population (Azer et al., 1999). Devitalized tissue is the site where the bacteria responsible for the non-healing ulcers inflict damage. Infectious agents are associated with amputation of the

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infected foot if not treated promptly (Logerfo et al., 1984). *Acinetobacter baumannii* has emerged as an important opportunistic Gram-negative bacteria in health care institutions globally, as it resists desiccation, is hard to eradicate and has numerous intrinsic and acquired mechanisms of drug resistance. Production of carbapenamases, which hydrolyse carbapenems, the drugs with high efficacy and broad spectrum of activity against this organism, has been a cause of worry to the clinician and the microbiologist (Peleg et al., 2008).

Carbapenamases are β-lactamases, which include serine-β-lactamases (KPC, OXA, GES, etc.) and metallo-β-lactamases (MBLs). The latter require metal ion zinc for their activity, which is inhibited by metal chelators like EDTA and thiol-based compounds but not by sulbactam, tazobactam and clavulanic acid (Karthika et al., 2009).

MBL production is typically associated with resistance to aminoglycosides and fluoroquinolones, further compromising therapeutic options. Among the seven types of MBL genes described throughout the World, bla_{IMP} and bla_{VIM} are the most common (Karthika et al., 2009).

The genes responsible for MBL production may be chromosomal or plasmid mediated and pose a threat of horizontal transfer. The introduction of carbapenems into clinical practice was of great help in the treatment of serious bacterial infections caused by β-lactam resistant bacteria and is the drug of choice for the infection caused by cephalosporin-resistant Gram negative infections (Varaiya et al., 2008).

The aim of the present study was to detect the prevalence of MBL producers among carpabenem-resistant *A. baumannii* strains isolated from diabetic foot ulcers with antimicrobial susceptibility pattern of *A. baumannii* strains isolated from diabetic foot ulcers.

MATERIALS AND METHODS

Over a one-year period from June 2012 to May 2013, 99 isolates of *A. baumannii* were isolated from patients with diabetic foot ulcers admitted to surgery department in Tanta University Hospital. The specimens were collected, transported and processed in microbiology laboratory without any delay. This work had the approval of the ethical committee in Faculty of Medicine, Tanta University and a written consent from all participants.

Inclusion criteria

Diabetic patients with duration of 10-15 days of diabetic foot infections, with no antibiotic therapy for one week before the study were used.

Exclusion criteria

Patients treated with antibiotics before admission for this attack of infection and chronic diseases other than diabetes.

Microbiological study

Culture specimens were obtained at the time of admission, after the surface of the wound had been washed vigorously with saline, and followed by debridement of superficial exudates. Specimens were obtained by scraping the ulcer base or the deep portion of the wound edge with a sterile curette. The soft tissue specimens were promptly sent to the laboratory for microbiological study (Shanker et al., 2005).

The isolates of *A. baumanni* were identified by conventional culture methods which are confirmed by biochemical reactions. Antimicrobial sensitivity testing was performed on Mueller-Hinton agar plates with commercially available discs (Hi-Media, Mumbai) by disc diffusion method. The results were recorded and interpreted as per Clinical and Laboratory Standard Institute (CLSI) recommendations (CLSI, 2012). The routine antibiotic sensitivity tests were put up for amikacin (30 µg), gentamicin (10 µg), amoxycillin (10 ug), tobramycin (10 µg), cefoperazone (75 µg), cefepime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), ceftizoxime (30 µg), ciprofloxacin (5 µg), imipenem (10 µg), meropenem (10 µg), chloramphenicol (30 µg), piperacillin/tazobactam (100/10 µg) and colistin (10 µg). Isolates were considered to be carbapenem resistant when the zone of inhibition around imipenem and meropenem discs was ≤13 mm.

MBL production

Screening for the detection of MBL was done by disc potentiation test with EDTA-impregnated imipenem discs and EDTA-impregnated meropenem discs and confirmed by multiplex PCR (Hemalatha et al., 2005).

Disc potentiation test methods

Test organism was inoculated onto plates of Mueller-Hintonagar plate (opacity adjusted to 0.5 McFarland opacity standards). A 0.5-m EDTA solution was prepared by dissolving 186.1 g of disodium EDTA $2H_2O$ in 1000 ml of distilled water and adjusting it to pH 8.0 by using NaOH. The mixture was sterilized by autoclaving. Two 10-mg imipenem discs and meropenem discs were placed on the plate; 5 ml of EDTA solution was added to one of the disceach. The inhibition zones of the imipenem and imipenem-EDTA discs and meropenem and meropenem-EDTA discs were compared after 16-18 h of incubation at 35 8°C. An increase in the zone size of at least 7 mm around the imipenem-EDTA disc and meropenem-EDTA discs was recorded as an MBL-positive strain (Leek et al., 2003).

PCR

Detection of the *bla*-IMP and *bla*-VIM genes was carried out using primers as described by Mostachio et al. (2009). *bla*-IMP primers: *bla*-IMP-F (5′-GAATAGAATGGTTAACTCTC-3′) *bla*-IMP-R(5′-CCAAACCACTAGGTTATC-3′) and *bla*-VIM primers: *bla*-VIM-F (5′-GTTTGGTCGCATATCGCAAC-3′) *bla*-VIM-R(5′-AATGCGCAGCACCAGGATAG-3′) were used.

A total of 4-5 identical colonies of *A. baumannii* were re-suspended in 500 µl of sterile saline in 1.5 ml Eppendorf tube. This was boiled at 100°C for 10 min, centrifuged at 8000 rpm for 5 min and the supernatant containing DNA was used for further processing. The PCR mixture used was as follows: 1 µl DNA template in a 49 µl mixture containing 10 mMTris/HCl (pH 8.8), 50 mMKCl, 4 mM $MgCl_2$, 200 µM each dNTP (FermentasGenetix Biotech Pvt. Ltd., New Delhi), 1 µl of each of the forward and

Table 1. Demographic and clinical characteristics of patients with diabetic foot infections.

Patients characteristic	No. (%)
Sex: Male	51 (51.51%)
Sex: female	48 (48.48%)
Age (years) Mean ± SD	49 ± 16.8
Clinical characteristics	
Duration of DM (years) Mean ± SD	12 ± 10.2
DM foot duration (days) Mean ± SD	12 ± 10.2
Duration of hospital stay	22 ± 5.58
Medications:	
OHA	60
Insulin	33
Wagner's classification:	
W1	34 (34.3%)
W2	21 (21.4%)
W3	15 (15.6%)
W4	19 (19.04%)
W5	Nil
Diabetic compliations	
Peripheal neuropathy	49 (49.35%)
Nephropathy	18 (18.44%)
Retinopathy	15 (15.22%)
Coronary artery disease	17 (17.32%)
Treatment	
Antibiotic alone	75
Surgical intervention	24

Table 2. Antibiotic sensitivity pattern of *A. baumanni* strains isolated from diabetic foot ulcers.

Antibiotics	Sensitive no. (%)
Amikacin	65 (65.6)
Gentamicin	13 (13.13)
Tobramycin	14(14.14)
Cefoperazone	9(9.09)
Cefepime	16 (16.16)
Ceftazidime	11 (11.1)
Ceftriaxone	13 (13.3)
Ciprofloxacin	12 (12.2)
Piperacilline/tazobactam	23 (23.33)
Imepenem	76 (76.5)
Meropenem	74 (74.4)
Amoxycilline	25 (25.4)
Colistin	43 (43.4)
Chloramphenicol	24 (24.4)

reverse primers (Bangalore genei) and 1 unit Taq DNA polymerase (FermentasGenetix Biotech Pvt.Ltd.,New Delhi). The PCR conditions included: initial denaturation at 94°C for 5 min followed by 33 cycles each of 94°C for 25 s, 53°C for 40 s and 72°C for 50 s, followed by a single final elongation step at 72°C for 6 min. The PCR product of 188 bp for *bla*-IMP and 382 bp for *bla*-VIM was visualized by 1.5% agarose gel electrophoresis containing ethidium bromide, 0.5 µg/ml (Bangalore Genei).

RESULTS

Out of 290 cases of diabetic foot ulcers attending surgery department, Tanta university hospital; 99 *A. baumannii* strains were isolated and were eligible for the study including 51 (51.51%) male patients and 48 (48.48%) with mean age±SD (49 ± 16.8 years). The mean duration of diabetes in the patients of the study was 12 ± 10.2 years and the mean duration of diabetic foot ulcers was 12 ± 10.2 days. Table 1 shows demographic and clinical characteristics of patients of the study.

The result of the study shows that 26 (26.26%) *A. baumanii* strains out of 99 strains were resistant to at least one or both carbapenem tested. Out of these, 22 (84.61%) were resistant to both imipenem and meropenem, 3 (11.53%) to meropenem and one strain (3.84%) to imipenem alone. Among 99 *A. baumanni* strains isolated from diabetic foot ulcers, Amikacin showed the highest level of sensitivity (65.6%), followed by colistin (43.3%). 9.09% only were sensitive to cefoperazone, 23.33% were sensitive to piperacillin/tazobactam. Table 2 shows antibiotic sensitivity pattern of *A. baumanni* strains isolated from diabetic foot ulcers.

The results of the study show that out of 26 carpabenem resistant *A. baumannii* strains 9 strains (34.61%) were positive for MBL by potentiated disc test, and 6 strains (23.07%) were positive for bla_{VIM} or bla_{IMP} by multiplex PCR; where bla_{VIM} gene was detected in 4 strains (15.38%) and bla_{IMP} was detected in 2 strains (7.69%). Figure 1 shows Agarose gel electrophoresis showing positive amplification of 382 and 188 base fragments specific for bla_{VIM} and bla_{IMP} respectively.

DISCUSSION

Diabetic foot ulcer is the most common complication requiring hospitalization among diabetic patients (Logerfo et al., 1984 and Bridges et al., 1994). It is also the most common cause of non-traumatic lower extremity ampu-tations (El-Tahawy et al., 2000). Physicians have an

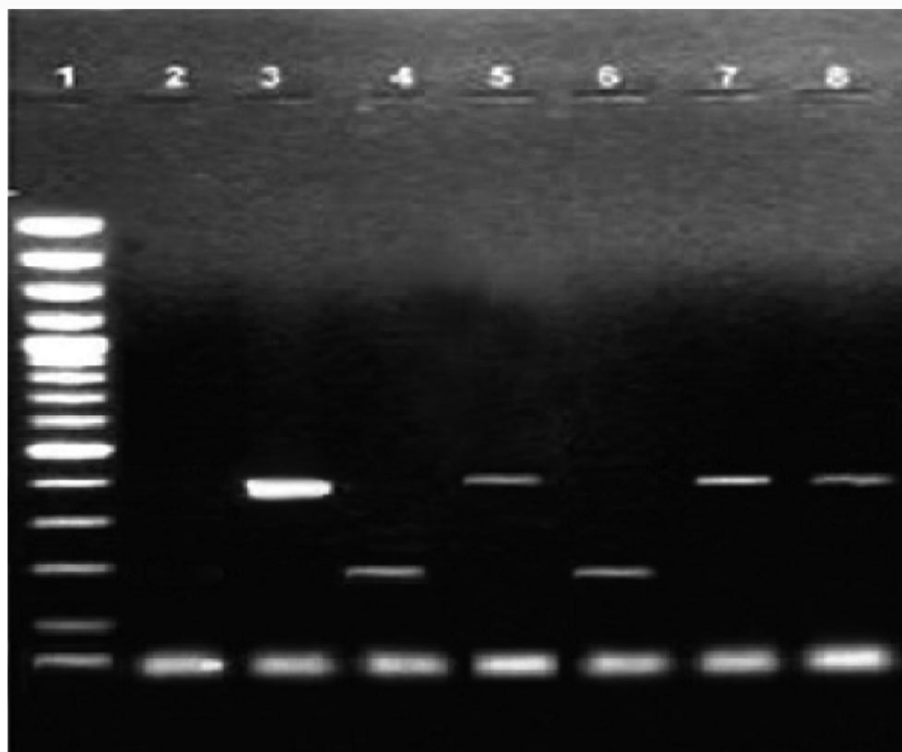

Figure 1. Agarose gel electrophoresis showing positive amplification of 382 and 188 base fragments specific for *bla*VIM and *bla*IMP, respectively. Lane 1, 100-1500 bp ladder; Lane 2, negative control; Lane 3, control *bla*VIM (382 bp); Lane 4, control *bla*IMP (188 bp). Lane 6, test strain (188 bp); Lanes 5, 7 and 8, test strain (382 bp).

important role in prevention, early diagnosis, and management of diabetic foot complications. Management, however, entails an extensive knowledge of the major risk factors for amputation and preventive maintenance with special reference to drug resistance in bacteria (Logerfo et al., 1984; El-Tahawy et al., 2000).

MBLs have been identified from clinical isolates in members of *Enterobacteriaceae, Pseudomonas aeruginosa* and *Acinetobacter* spp. over the past few years (Walsh et al., 2005; Kohlenberg et al., 2009). Strains producing these enzymes have been responsible for nosocomial outbreaks that have been accompanied by serious and prolonged infections. MBLs are powerful carbapenemases and can hydrolyze a wide variety of β-lactams, including penicillins, cephalosporins and carbapenems. Since the initial isolation of carbapenem resistant *A. baumannii* producing *bla*-IMP-1 and *bla*-VIM-1 type MBL in Japan and Italy, respectively (Lee et al., 2003). Clinical isolates of these strains have been identified worldwide. CLSI document (2009) has no guidelines for detecting MBLs, however, it has recommended modified Hodge test for detection of carbapenamases but in members of *Enterobacteriaceae* only.

The present work was carried out over a period of one year that extended from June 2012 to May 2013 on 290 patients with diabetic foot ulcers attending department of surgery in Tanta University Hospital. Out of 290 clinical samples, *A. baumanni* strains could be isolated from 99 (34.13%) cases. This percentage was in agreement with Gadepalli et al. (2006) who could isolate *A. baumanni* from 40% of cases of diabetic foot ulcers.

The results of antimicrobial susceptibility profile for the 99 *A. baumanni* strains isolated in this study showed that the highest sensitivity was to Meropenem (74.4%), Imepenem (76.55%), Amikacin (65%) and the lowest sensitivity was to Cefoperazone (9.09%), Ceftazidime (11%) and Ciprofloxacin (12%). The results showed that 9 strains (9.09%) were only sensitive to cefoperazone, and 23 strains (23.33%) were sensitive to piperacillin/-tazobactam. The study of Umadevi et al. (2011) showed that the majority of *Acinetobacter* spp. were susceptible to piperacillin-tazobactam (83%), imipenem (67%) and trimethoprim-sulfamethoxazole (67%), while being less susceptible to gentamicin (17%), amikacin (50%), ciprofloxacin (67%), tetracycline (50%), ceftriaxone (33%) and ceftazidime (33%). In accordance with the results of this study, the result of Shanker et al. (2005) showed that

76.4% of the isolated *A. baumanni* strains in their study were sensitive to Amikacin, but in reverse to the results of this study they found that 100% of the isolated *A. baumanni* strains were sensitive to Imepenem and ciprofloxacin and 76.5% of the strains were sensitive to piperacillin/tazobactam

Carbapenems are used for treating serious infections caused by multidrug-resistant Gram-negative bacilli. Resistance to carbapenems is due to decreased outer membrane permeability, increased efflux systems, alteration of penicillin-binding proteins, and the production of carbapenem hydrolyzing enzymes, that is, carbapenemases. The resistance may also be due to the production of metallo-β-lactamases (MBL); such resistance can be chromosomally encoded or plasmid mediated (Gladstone et al., 2005)

The results of the present study showed that 26 (26.26%) *A. baumanii* strains out of 99 strains were resistant to at least one or both carbapenem tested. Out of these 26 carpabenem resistant strains, 22 (84.61%) were resistant to both imipenem and meropenem, 3 (11.53%) to meropenem only and one strain (3.84%) to imipenem alone. In various studies across the world, varying rates of resistance (4-60%) have been reported for imipenem and meropenem. Among the Indian workers, Gladstone et al. (2005) reported 14.2%, whereas Taneja et al. (2003) reported 36.4%.

As regard the EDTA-imipenem-microbiological assay, which differentiates metalloenzymes from non-metallo-enzymes, in *A. baumanii* strains, by this assay, we could confirm 9 isolates (9.3%) as MBL producers and 15 (32.56%) as non-metalloenzyme producers among the 26 screen test positives. However, of the 9 MBL positives only 4 (15.38%) showed presence of *bla*-VIM and 2 showed presence of *bla*-IMP (7.69%) and none of the 15 non-metalloenzyme producers showed presence of either *bla*-IMP or *bla*-VIM. Karthika et al. (2009) who found *bla*-VIM MBL gene only in 7 (16.28%) of the 43 screen test positive isolates reported *bla*-IMP-1 in 42% of *A. baumannii.* while *Amudhan* et al. (2001) reported *bla*-VIM in 46.55% with and both *bla*-IMP and *bla*-VIM in only one isolate of *A. baumannii*. The non-demonstration of *IMP* and VIM genes in 20 of our 26 screen test positive isolates could be either due to presence of unidentified MBL gene, limitation of the primer set used either with regards to picking up the variant IMP/VIM gene or because of presence of MBL genes other than IMP/VIM, presence of other enzymes [OXA like (Ambler class D) carbapenamases AmpC β-lactamases] or other mechanism of carbapenem resistance, namely; loss of porins, increase in efflux pump activity alteration in penicillin binding proteins (PBPs), Singh et al. (2009). "False" positivity of disc potentiation test method (three isolates) against the PCR, could be due to presence of either other uncommon MBL encoding gene like SIM, SPM, GIM, AIM or variants of IMP and VIM (Singh et al., 2009). Very high MBL positivity of 95.2 and 88% in *P. aeruginosa* using disc potentiation test method has been reported by Singh et al. (2009) and Jain et al. (2011), respectively. However, Quinones-Falconi et al. (2009) using PCR as a gold standard reported only 3.5% MBL positivity in *P. aeruginosa* using disc potentiation test method. Galicia et al. (2009) have reported 3.4% MBL positivity in *P. aeruginosa* using disc potentiation test method with excellent specificity but poor sensitivity of the test. We did not come across any study on EIM in *A. baumannii*

Conclusion

Rapid dissemination of carbapenem-resistant isolates in diabetic foot ulcers is worrisome and calls for judicious use of antibiotics .The. bla_{VIM} and bla_{IMP} genes have a role in carbapenem resistance in the community. More studies are needed to differentiate MBL from non-metalloenzymes producers.

ACKNOWLEDGEMENT

The authors greatly acknowledge Medical Microbiology and Immunology, Tropical Medicine, Internal medicine and surgery Departments, Faculty of Medicine, Tanta University for their great support and co-operation.

Conflict of Interests

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

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Vol. 8(15), pp. 1586-1600, 9 April, 2014
DOI: 10.5897/AJMR2013.6062
Article Number: 9F66F6343822
ISSN 1996-0808
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African Journal of Microbiology Research

Full Length Research Paper

Attempt of simulation of models to predict the disinfection efficacy of an UV disinfection reactor and kinetic study of inactivation of selected bacteria of *Pseudomonas aeruginosa* in a laboratory UV device

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Received 17 June, 2013; Accepted 17 March, 2014

The aims of this paper were to propose a modeling system of water UV disinfection, establish the influence of UV doses on the kinetics of disinfection, study UV-resistant strains of *Pseudomonas aeruginosa,* and improve the performances of this multipart process. The UV disinfection should inactivate pathogenic microorganisms and improve the hygienic quality of water. A comprehensive treatment in considering the mathematical aspect to model the UVc disinfection of water was achieved. A complete mathematical description of the inactivation kinetics is developed and showed two successive stages, a fast and a low one. Similarly, a mathematical model describing fluid flow and concentration of the microorganisms inside a UV reactor is developed. Modeling the kinetic and the UV lamp ray emission using some empirical approaches might increase the efficiency of UV disinfection and improve its performance. This study shows an improvement of the microbial inactivation rate of about 49% for the selected pathogenic resistant bacteria of *P. aeruginosa* (S3), and in considering perfectly mixed water flowing into the UV reactor.

Key words: Disinfection, UV_{254}, modeling, pathogenic microorganisms, performance.

INTRODUCTION

Disinfection of potable and wastewater using additives like chlorine, ozone or silver has a long tradition (Christoph, 2006). However, these treatments can result in the formation of disinfection by-products which are harmful to humans (Oparaku et al., 2011). Additionally, certain microorganisms are particularly resistant to chemical disinfection. Treatment with UV radiation offers a way out, since it does not involve chemicals, producing very few by-products as compared to chemical methods, while not altering taste or chemical composition of the water. For this reason, water treatment with ultraviolet radiation becomes increasingly important and has received wide recognition as an important contribution to the protection of public health (Weinberg et al., 2002). The knowledge acquired in this field demonstrates that the use of UV for disinfection is a fast, efficient, safe and cost-effective process (Meiting et al., 2009). It has been used for many years in several countries to disinfect the

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water (USEPA, 2003b; Clancy et al., 2000).

Disinfection reactors that employ ultraviolet (UV) radiation inactivate pathogenic microorganisms by altering their genetic material, thereby hindering subsequent replication. The efficiency with which UV disinfection reactors are able to inactivate microorganisms is dependent on the hydraulic characteristics of the reactor, the fluence rate distribution within the reactor, and microbial inactivation kinetics or UV dose-response behavior of the target microbial pathogen(s) (Zorana et al., 2008). However, microorganisms have evolved repair mechanisms and can reactivate, once their DNA is partially denatured. Visible light and time may have a positive influence on this process known as reactivation. UV light is electromagnetic radiation, which disinfects water by damaging the genetic material of microorganisms.

Lesions in the form of pyrimidine dimers are induced in the genomic DNA or RNA of microorganisms (Sinha and Häder, 2002), preventing normal replication, which effectively inactivates exposed microorganisms. This is justified when the water to be treated must fulfill certain things to obtain an optimal effect of UV irradiation. Physicochemical parameters such as water turbidity, hardness, suspended solids, iron, manganese, humic acids are disruptive factors of UV disinfection. Substances in water weaken the transmission rate, and deposits may also tarnish the UV reactor and taints the tubes of quartz protecting the UV lamp (Sellami et al., 2003). The regular change and cleaning of UV quartz sheath lamps provide a good ray permeability thus good distribution. Besides, several parameters can also influence the rate of microbial inactivation such as the UV dose applied, the stability of disinfectant, the contact time, the pH and the temperature of water, and the number and type of microorganisms in water (in terms of resistance) (Hassen et al., 1997). The relationship between these parameters can be evaluated by means of analytical measurements in the laboratory.

However, because of the complex dynamics governing this process, only certain parameters such as the UV dose, the contact time and the content of suspended solids in water can be studied at laboratory scale to establish laws monitoring the UV disinfection. To better explain the process of inactivation, some disinfection kinetic models have been proposed in the literature to validate the experimental results, beginning from the simplest model UV water purification lamps produce UV-C or germicidal UV, with radiation of much greater intensity than sunlight. Almost all of a UV lamp's output is concentrated in a 254 nm region in order to take full advantage of the germicidal properties of this wavelength. Most UV purification systems are combined with various forms of filtration, as UV light is only capable of killing micro-organisms such as bacteria, viruses, molds, algae, yeast and oocysts such as Cryptosporidium and Giardia. UV light generally has no impact on chlorine, volatile organic compounds (VOCs), heavy metals and other chemical contaminants.

Nevertheless, it is probably the most cost-effective and efficient technology available to homeowners to eliminate a wide range of biological contaminants from their water supply. This study was therefore carried out to investigate the effectiveness of UV light for wastewater disinfection (Oparaku et al., 2011).

This is justified when the water to be treated must fulfill certain physico-chemical conditions to get a good effect of UV irradiation. Physico-chemical features such as water turbidity, hardness, suspended solids, iron, manganese, humic acid content are disruptive causes of UV disinfection. Substances in water weaken the transmission rate, and deposits may also tarnish the UV reactor and taints the tubes of quartz protecting the UV lamp (Sellami et al., 2003). The regular change and cleaning of UV quartz sheath lamps provide a good ray permeability thus better distribution. Besides, several parameters can also influence the rate of microbial inactivation such as the UV dose applied, the stability of disinfectant, the contact time, the pH and the temperature of water, and the number and type of microorganisms in water (in terms of resistance) (Brahmi et al., 2013). The relationship between these parameters can be evaluated by analytical measurements in the laboratory. However, because of the complex dynamics governing this process, only certain parameters such as the UV dose and the contact time can be studied at laboratory scale to find out laws monitoring the UV disinfection.

On the other hand, the optimization of the process of water disinfection by UV irradiation can be tried by several approaches. The first is a technological approach that concerns the choice of the different units of the system; the second is an economic approach that concerns optimisation of the costs. The mathematical approach is helpful in modeling and lessens expensive experiences by reducing the operation of controls.

The biotechnical modeling presents great difficulties. Indeed, it needs to involve a multidisciplinary group of experts in biology, chemistry, mathematics, automation, etc. Whereas, it is to be realized that, even if the models are established, the process is likely to be complex.

This research was aimed at first, understanding and evaluating the germicidal UV water disinfection, secondly to establish the influence of UV doses on the kinetics of disinfection, to study UV-resistant strains of *Pseudomonas aeruginosa*, and thirdly to establish and diagnose a mathematical model for simulation and improvement of the UV_C water disinfection.

MATERIALS AND METHODS

Types and characteristics of treated wastewater used

The treated wastewater samples used in this study were collected at the outlet of a pilot wastewater treatment plant (WWTP)

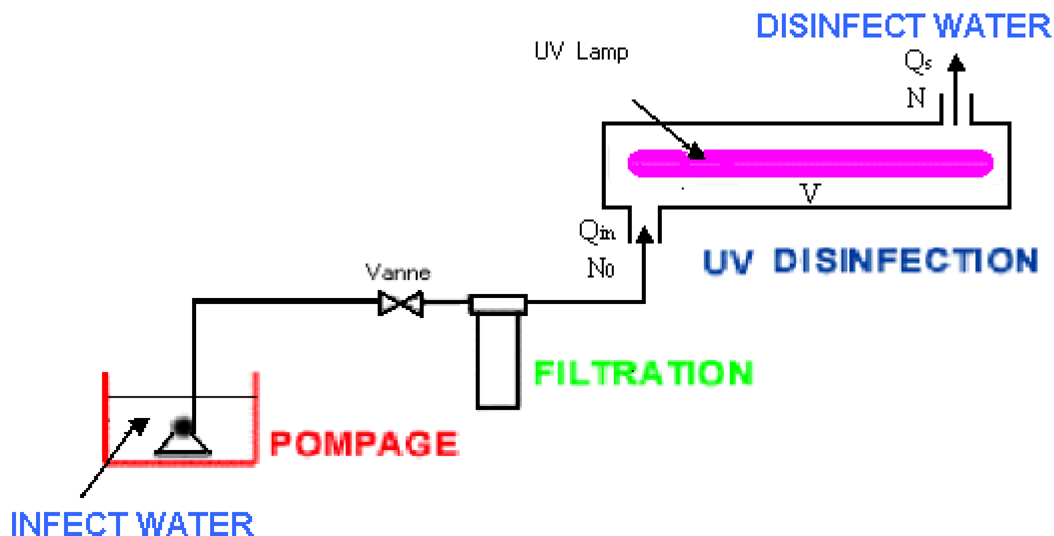

Figure 1. Synoptic diagram of the UV pilot scale disinfection system.

belonging to the Water Research and Technology Center, Tunisia. The pilot WWTP is connected to the sewerage network of the city of Tunis that includes a process capacity of 150 m^3 per day. It is composed of 4 treatment lines operative in parallel: a trickling filter, rotating biological discs, and a land and lagoon optional filter. Throughout disinfection tests, the physic-chemical characteristics of the wastewater treated by the trickling filter did not show considerable change. The values fluctuated between 47 and 49% for ultraviolet light transmission, 15 to 47 mg/L for total suspended solids (TSS), 20 to 29 mg/L for biochemical oxygen demand (BOD_5) and 90 to 102 mg/L for chemical oxygen demand (COD).

Experiments in a batch laboratory irradiation device

The laboratory UV device used in this study has previously been described by Hassen et al. (1997). A low pressure UV-C lamp is used. This lamp emitted an average intensity of about 7 $mW.cm^{-2}$. In addition, all bacterial strains of *P. aeruginosa* studied were cultivated to a mid-log phase at 37°C in 20 mL of nutrient broth. Each culture was centrifuged at 5,000 rpm/min for 15 min and the pellet was washed twice with sterile distilled water. The washed pellet was resuspended in 10 mL sterile distilled water. Test organisms were then seeded separately, into 20 mL of sterile wastewater with UV transmittance of 50%, to give a viable cell count of approximately a 10^5 to 10^6 colony-forming unit (CFU)/mL, the same mean count as that in the secondary wastewater suspension. The test organisms were then exposed to the UV-C light for various times ranging from 2 to 90 s.

UV pilot equipment

This study was carried out in a wastewater treatment pilot plant equipped with a monolamp UV reactor supplied by Katadyn (Katadyn Produkte AG, Wallisellen, Switzerland). This UV reactor has a useful volume of 2 L and constituted a stainless cylindrical container ran continuously during the study. A low pressure mercury vapor discharge lamp (length = 680 mm, diameter = 18 mm, power of UV emission at 254 nm = 65 W) was inserted into a quartz sleeve for mechanical protection and sealing. Every month the sleeve was cleaned mechanically with a dilute hydrochloric acid solution to prevent a filthiness of the lamp. A selective detector for UV (253.7 nm) joined to a radiometer (Vilbert- Lourmat, Norme la Vallée, France) allowed the measure of UV intensity at the emerging of the quartz sleeve. The applied UV dose (or fluence) is traditionally characterised in terms of the energy per surface area or $mJ.cm^{-2}$, and is a product of the average UV intensity (fluence rate) ($mW.cm^{-2}$) multiplied by the exposure time in seconds. UV is attenuated by UV-absorbing substances in water, which reduce the transmittance of UV through water and consequently impact the dose received by microorganisms. The UV transmittance of water to be disinfected is therefore taken into account when calculating the average UV intensity applied to water. The synoptic diagram of the UV pilot-scale disinfection is represented in Figure 1.

Costache et al. (2001) showed that it should be better in real conditions of the UV disinfection to supply the lamp with the strongest possible electric current. The schematic concept of the UV irradiation is represented in Figure 2.

A decrease of the water debit at the entry of UV reactor is linked to an improvement of the removal of pathogenic microorganisms. Theoretically, the time of UV exposure can vary from 1 to 300 s, but often a time of exposure of 7 to 15 s proved to be enough to guarantee a satisfactory abatement (Hassen, 1998).

For efficient water disinfection, it is essential that all parts and each volume of the water receives sufficient UV ray exposure of at least 88 $mW.s.cm^{-2}$ (at 253.7 nm and water transmission superior to 45%) to reduce human pathogens (fecal streptococci and fecal coliforms) by at least 3 logs (Hassen et al., 2000). The homogeneity of the flow pattern and the radiation field may have critical effects on disinfection.

Bacterial strains selected for UV-disinfection study

Many pathogens are responsible for waterborne diseases. Despite the development of molecular methods, currently it is not always possible to detect comprehensively all micro-organisms in a water sample. Therefore, most studies in this area have mainly focused on the number of fecal indicator bacteria (total coliforms, fecal coliforms and fecal streptococci in general) to estimate the population of pathogens. However, recent studies showed that the species of *P. aeruginosa* seems to be a valid sanitary indicator for recreational waters (Brahmi et al., 2010). This parameter is actually

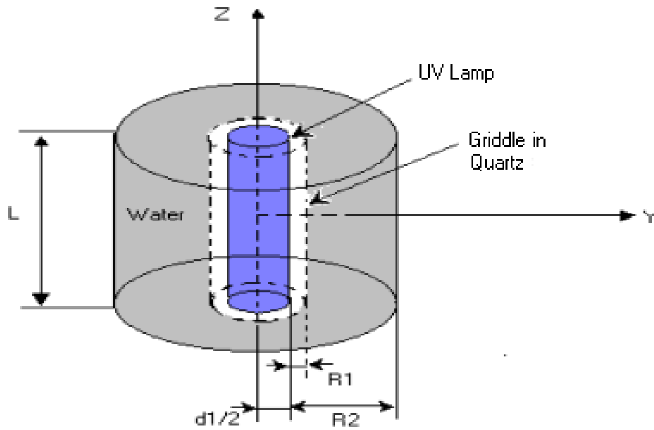

Figure 2. Configuration of the UV irradiation disinfection system. Width: L, d1, R1 and R2 are length of the reactor approximated to the length of lamp, diameter of the lamp, radius of the quartz sleeve and the interior radius wall of the irradiation room, respectively.

used as a criterion in the regulation of wading and swimming pools. Moreover, the absence of *P. aeruginosa* is important not only for its role as an indicator, but also because it is an opportunistic pathogen of which the transmission is often associated with water. Its use for evaluating the effectiveness as a treatment of UV-disinfection seems therefore reliable. Consequently, its kinetics of inactivation by UV irradiation has assumed the same fate as for all other less resistant pathogens.

For all the above reasons, a collection of three strains of *P. aeruginosa* includes strains of *P. aeruginosa* ATCC 15442 [PA = S1] (provided by DIFCO, laboratory POBOX 331058, Detroit M 48232-7058 USA). The other two strains were isolated from wastewater and treated without a repetitive sequential dose of UV (S2 and S3). All the strains were grown in the laboratory in nutrient broth (Institute Pasteur Production).

On the other hand, we defined the UV-C dose received by the microbial cells as the product between the time of exposure (sec) to UV-C and the intensity emitted by the UV lamp ($mW.cm^{-2}$).

The kinetic models used for UV-C inactivation

These kinetic approaches are based on experimental studies using: a laboratory disinfection device; 3 selected strains of *P. aeruginosa* grown on a nutrient agar (Pasteur Institute Production, Tunisia); and different simulation models, from the simplest model of Chick-Watson reduced to first-order kinetics, to complex models such as the modified Chick-Watson model.

The model of Chick-Watson is used primarily to express the kinetics of disinfection with chemical disinfectants (Trussell and Chao, 1977; Roustan et al., 1991). The first-order kinetics is expressed as follows:

$$\frac{dN}{dt} = -K \times C^{n} \times N \quad (1)$$

The integration of this expression gives:

$$N/N_0 = e^{-KC^{n}t} \quad (2)$$

C is the concentration of disinfectant used; *K* is a coefficient reflecting the specific case of disinfecting lethality potential; *n* is the coefficient of dilution, which is a function of disinfectant and pH of water (the value of *n* is usually close to unity); and *t* is the exposure time to disinfectant.

In the case of UV-disinfection, an amendment to this model was made by replacing the concentration of chemical disinfectant (*C*) with the intensity of UV radiation, as proposed by Haas (1999). The disinfection kinetics could be rewritten as follows:

$$dN/dt = K \times I^{n} \times N \quad (3)$$

The integration of this expression gives

$$N/N_0 = e^{-KI^{n}t} \quad (4)$$

Changing the logarithmic form and using a linear regression, the kinetic parameters (*K* and *n*) of the latter expression could be determined as follows:

$$Ln\left[-Ln\left(N/N_0\right)\right] = Ln(K) + n.Ln(I) + Ln(t) \quad (5)$$

When $n < 1$, the disinfection process is more controlled by the contact time than by the UV dose. When $n > 1$, the UV dose takes precedence over the contact time in the control of the process (Leahy et al., 1987).

RESULTS AND DISCUSSION

Behavior of *P. aeruginosa* strains after UV irradiation

P. aeruginosa strains issued from an environmental origin (S2 and S3) tested were isolated from wastewater and submitted to a sequential and alternate treatment of 2 or 4 min of exposure to UV rays, called passage. These successive passages of 2 or 4 min exposure to UV_{254} rays were performed in 90 mm Petri dishes. After each passage, the environment is enriched by a solution of 5 ml of asparagine (5 g/l) and incubated for 1 h at 37°C. Both strains tested S2 and S3 were exposed to 34 and 86 passages, respectively, and corresponding to a cumulative UV dose of 68.544 and 173.376 $mW{\cdot}s{\cdot}cm^{-2}$, respectively.

Thus, we recorded a significant resistance to UV radiation for these two strains marked by good growth and intense pigmentation (release of pyoverdin or fluorescein known as a specific fluorescent pigment released by these species in some definite circumstances).

In this sense, a study published by Hassen et al. (1997) and recently by Lesavre and Magoarou (2004), showed that the treatment of *P. aeruginosa* strains with low UV doses (<30 $mW{\cdot}s{\cdot}cm^{-2}$) has a significant effect on the growth stimulation of these bacteria. To confirm the acquisition of UV resistance of these two strains (S2 and

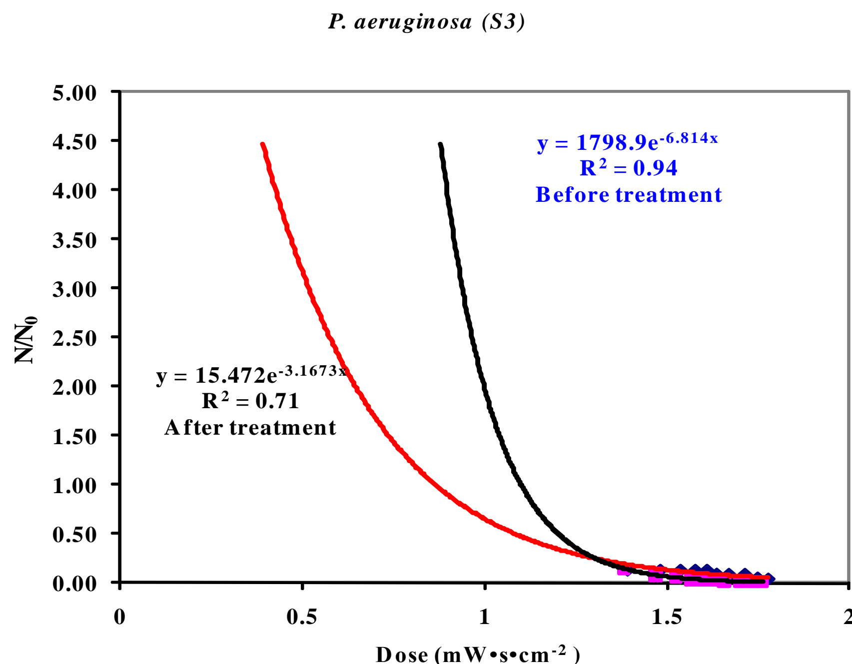

Figure 3. Inactivation of *P. aeruginosa* (strain S3 for example) according to UV dose applied before and after a prolonged exposure to UV ray. N/N_0 : Rate of inactivation of microorganisms after exposure to UV; *N*: Number of bacteria after the period of exposure to laboratory light; *N*0 : Number of microorganisms at the instant $T = 0$ ($N_0 = 10^7$ organisms/100 ml).

S3), a kinetic inactivation study was carried out and an example of the experimental results obtained for the strain S3 is shown in Figure 3. As evident from the figure, the kinetics abatement of the strain S3 treated with UV differ significantly from that of the S3 strain of departure (not treated). Therefore, the sequential UV treatment of the starting strains S3 induced a significant resistance to UV radiation.

Inactivation kinetic of *P. aeruginosa*: UV dose-response

Several mathematical relationships have been developed to describe bacterial responses to UV irradiation. UV dose plays an important role in all bacterial inactivation models for UV irradiation (Qualls et al., 1989).

In this study, the curve commonly illustrating the kinetics of inactivation usually showed a significant gap between the experimental points and those simulated by the model in the case of studied strain S2 of *P. aeruginosa* taken as a model (Figure 4a). In the same way, the determination of sum of squares of residuals (SSR), a representative parameter of the difference between the experimental values $(N/N_0)_{mes}$ and the calculated values of the model $(N/N_0)_{Cal}$, appeared to be important to this strain ($R^2 = 0.60$, SSR = 0.17). Therefore, we found that the model of Chick-Watson, reduced to a first-order kinetic with $n = 1$, showed its limits, and that the inactivation process is most often non-uniform, and does not necessarily comply, as first-order kinetics, with an exponential law (Haas, 1999; Hassen, 1998; Shayeb et al., 1998). However, the adopted experimental protocol showed a very noticeable reduction rate for low doses of irradiation. The importance of UV radiation intensity of the lamp allows a yield rate of 2 U-log to be achieved after only 2 s of exposure.

A decrease in additional U-log could not be attained, even after an exposure time of 90 s (results not shown). The application of a first order kinetic during the second stage involves the change of the model by introducing a dimensionless coefficient A, in order to reflect the decline achieved during the first fast kinetics stage (Figure 4b).

This initial abatement *A* is suggested and added to the common Equation 3 of the model to view and validate this empirical model at first and secondly to reflect the decrease achieved during the first fast kinetics stage. The expression of the model (4) becomes (Mamane-Gravetz and Linden, 2004):

$$\frac{N}{N_0} = A.\exp(-kIt) \quad (6)$$

Where *A* is the initial abatement of the number of microorganisms during the contact between UV rays and

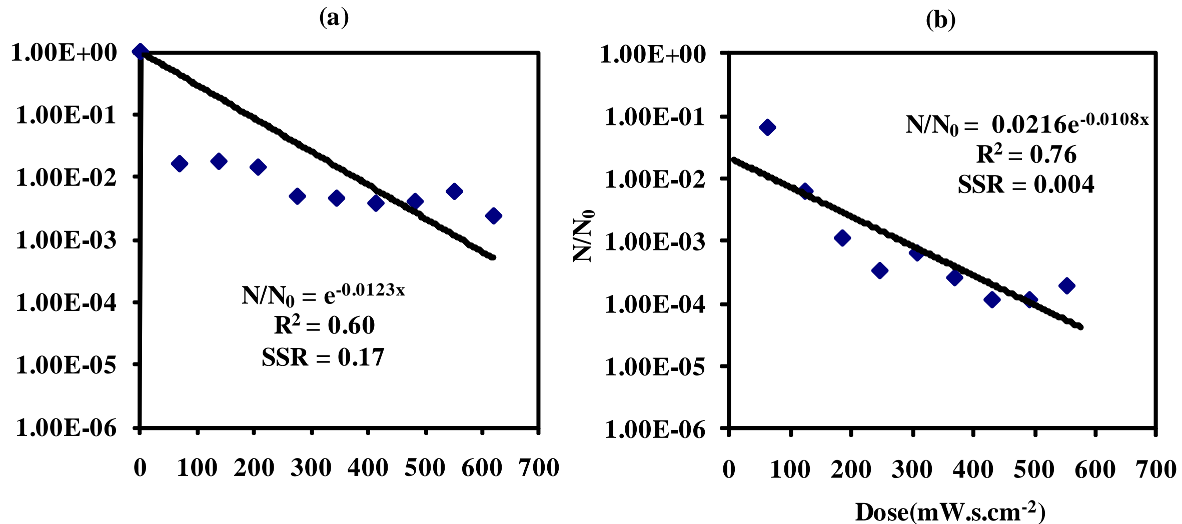

Figure 4. Study of the disinfection efficiency as a function of irradiation dose according to the models of Chick-Watson (a) and Amended chick-Watson (b), respectively. y: reduction = N/N_0 with N; number of bacteria at the instant T; N_0; Number of bacteria at the instant T= 0; R^2: Coefficient of determination; Dose (mW.s.cm^{-2}) = $I \times T$= UV intensity (mW.cm^{-2}) × time of contact (s).

Table 1. Some examples of kinetic inactivation according to the modified Chick-Watson model.

Strain	Equation 1 (y_1)	Equation 2 (y_2)
P. aeruginosa ATCC15442 *(S1)*	y = 0.0757exp(-0.0361It)	y = 0.03 exp(-0.0167It)
P. aeruginosa (S2)	y = 0.0381exp(-0.0297It)	y = 0.0065 exp(-0.0057It)
P. aeruginosa (S3)	y = 0.0611exp(-0.0163It)	y = 0.0089 exp(-0.0018It)

water. The parameters to identify in this case are K and A.

By working out the difference in these two cases (*SSR and* R^2), the values obtained depending on the model of Chick-Watson in its modified form were smaller (*SSR= 0.004*) than those calculated using the same model in its initial form (*SSR= 0.17*). In the same way, the correlation coefficient R^2 obtained using the amended model of Chick-Watson were generally higher (R^2= *0.76*) than those obtained using the same model in its original form (R^2= *0.60)* (Figure 4). Thus, we found that the adjustment of the same model when considering an initial reduction describes quite well the kinetics of disinfection for most of the studied strains.

On the other side, the results of Sellami et al. (2003) showed that the first instants of UV exposure (2 to 10 s) are capital for bacterial inactivation where the UV lethal effect is critical and destructive, and later added UV exposure would be of less damage to microorganisms, and surviving numbers slowly decline.

The end of the kinetic curve of UV inactivation has a tailing phase due to UV resistance of the microorganisms and to experimental conditions, such as suspended solids content, turbulence in the reactor, which may block the UV irradiation.

Indeed, the microbial inactivation speed is important in this first interval of time. The increase of the contact time with UV rays beyond this interval has no significant effect on inactivation, and the speed of bacterial inactivation becomes slow and constant. According to the UV dose applied, two types of inactivation prevail: a high rate of inactivation with weak UV doses, and a low rate of inactivation with fairly doses.

All these factors favour the idea of a kinetic multimodel of microbial inactivation: a first model with rapid dynamics that describes the kinetics of disinfection during the first instants of UV exposure, and a second model with slower dynamics. Table 1 shows examples of the two modes of UV inactivation according to the modified model of Chick-Watson (with the initial reduction A, equation 6) for some selected bacterial strains.

Each examined strain is characterized by two equations y_1 and y_2. For a given microorganism, the instant of encounter between the representative curves of equations (y_1) and (y_2) is often considered as an instant of commutation between the first high-rate dynamic (y_1) and the second low-rate dynamic model (y_2).

Figure 5 illustrates an example of the experimental results according to the curve of adjustment of the model.

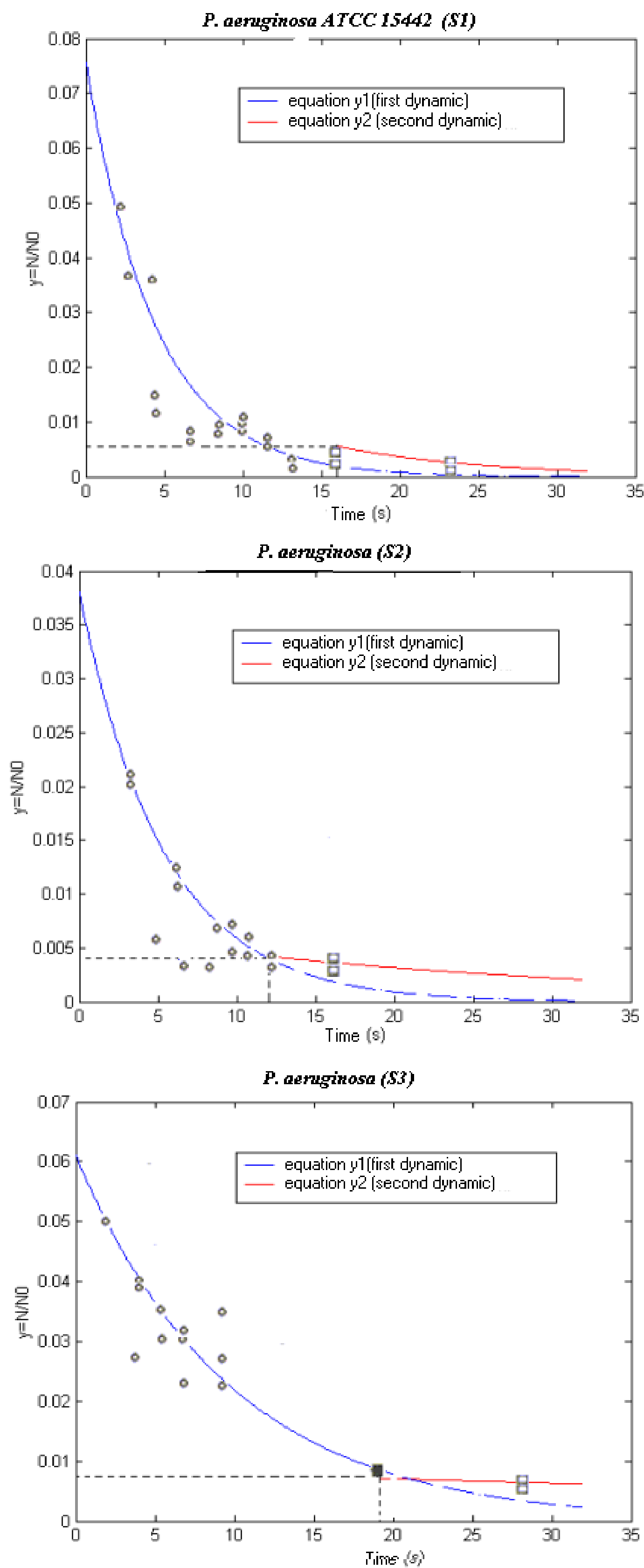

Figure 5. Some kinetic examples of microbial inactivation adjustment according to the modified Chick-Watson multimodel.

The variation of the kinetics of disinfection follows the UV irradiation dose. The switching between equations 1 (y_1) and 2 (y_2) depends on the genera and the type of microorganism, and on the amount of UV applied; and so, is dependent on time if the UV ray intensity is considered constant (I = Constant = 6.3 mW.s.cm^{-2}).

On the other hand, the experimental batch study reported by Hassen et al. (2000) using some selected bacteria, and the modified model of Chick-Watson showed that the kinetics of bacterial inactivation varied according to the UV dose applied. The first instants (2 to 10 s and corresponding to average doses of 10 and 80 mW.s.cm^{-2}) appeared as a controlling factor. It is assumed that outside the effect of dose, an effect of shock is needed to assure inactivation at least for certain microbial species.

Difficulties of modeling mercury low-pressure vapor discharges lamp

The energetic flux emitted by the mercury vapor lamp is a complex function depending basically on the two factors, electric current and time. Further, these functions depend on the geometric characteristics of the lamp, the rare gas used, the pressure within the lamp and the external temperature of the lamp. These variables are complicated and depend on the hysteretic and on the internal dynamic ones (dynamic conductance of the lamp) of which equations of variation are not precisely determined, and diagrams are no monotones. These factors complicate the development of the general model characterizing the variation of emitted flux or the arc tensions according to the arc current and time (Zitouni et al., 2011). Results and relationships between all these variables are determined using an empirical approach.

To determine a relationship between the electric powers consumed by a germicidal low-pressure mercury lamp, a bibliographic research showed that in a general way, 50 to 60% of the electric power consumed by the germicidal low-pressure mercury lamp is transformed into UV radiation. The rest is scattered by the effects of collision, excitation, diffusion, thermal conduction (Porras, 1998). The tension of the arc is the product of the arc current and conductance that depends on a nonlinear function of the current, which are the exact relationships under investigation.

The lack of a real function relating tension current or current-emitted flux, leads us to use the electric power consumed as follows:

$$F_0 = 0.55P \qquad (7)$$

Where, F_0 and P are the UV flux emitted and the electric power consumed by the UV lamp, respectively.

We estimated in Equation 7 that 55% of the electric power consumed is transformed into emitted flux. This value of 55% is often used in the literature (Porras, 1998; Sarroukh et al., 1999).

Optimization of the UV disinfection

Generally, the UV disinfection of water takes place with a variable flow and the most possible favorable surrounding conditions; in addition, biologists are expected to work with constant UV intensity (constant I) except for uncontrolled variations inherent to the environment, for example, temperature.

This study showed that temperature increase improved the effectiveness of UV irradiation emission and thus improved bacterial inactivation (Brahmi et al., 2012). As mentioned above, (i) the model of Chick-Watson modified with an initial reduction A represented correctly the experimental result (ii) the kinetics of UV inactivation include two types, the first is a rapid dynamic and high inactivation rate and the second a slow dynamic and low inactivation rate that stabilize through time. This approach allowed us to affirm the efficiency of UV irradiation decreases throughout the time. This decrease results to a constant UV irradiation during disinfection. The idea is to increase as much as possible the energy during the first instants of irradiation that are determinant for the bacterial inactivation, and in a second phase to lower the irradiation dose as the efficiency of the UV decreases.

Influence of water flowing on average UV irradiation

Water specialist researchers in general use a probe that measures UV irradiation on the surface of the quartz sleeve. This UV emission corresponds to the strongest radiation in the irradiation room if we ignore the effect of existing air between the quartz sleeve and the surface of the lamp. As shown above, the width of the water layer causes a serious decrease of UV radiation (optical path), and therefore the use of ray intensity (*I)* mentioned by the probe is not efficient for modeling the UV reactor. In this study we calculated the average radiation in the irradiation room. This average radiation depends physically on the water flow.

Indeed, there are two limit types of flow, the piston and the perfectly mixed. For piston flow, all fluid molecules cross the chamber of reaction at the same speed in parallel trajectories. In this layered or laminar flow, there is neither mixture nor dispersion; all particles have same residence time equal to the average time of hydraulic stay. The response is therefore the realistic reproduction of the excitation. For a perfectly mixed flowing, the entering fluid is scattered instantaneously in the entire volume of the reactor by an intense agitation. The average dose prevailing in the reactor is defined as the report between the total energy deposed per second in

the interior of the reactor and the fluid volume that crosses the entire reactor chamber per second. Although, the fluid volume depends on the speed of fluid circulation in the reactor, subsequently, calculation of the efficient radiation presume the speed of the fluid which is known. This speed depends on the type of circulation of the fluid in the reactor (Costache, 2001).

Case of piston flowing

We consider the case of reactor in the Figures 1 and 2, with a minimum and a maximum radius *R1* and *R2*, and a length *L*, without absorption and mixture, where the UV radiation intensity I_r depends on the distance *r* to the reactor axis. The speed of circulation in all points of the irradiation room (v_r) is constant and equal to the speed in the center of the reactor (v_0):

$$v_r = v_0 \tag{8}$$

In these conditions, we can write the fraction of flow submitted to a UV radiation as:

$$I_r = \left(1 - \lambda \frac{r}{R_2}\right) \frac{F_0}{2\pi r L} \tag{9}$$

Equation (11) is equal to

$$Q_r = \frac{1}{\pi\left(R_2{}^2 - R_1{}^2\right)} 2\pi r dr \tag{10}$$

Where λ represent a constant equal to 0.1 in this case, and F_0 = 0.55*P* the irradiation flux emitted by the source. Thus, the weighted average value of UV radiation intensity *I* of flow will is expressed as:

$$I_{Mp} = \int_r I_r Q_r \tag{11}$$

Or more,

$$I_{Mp} = \frac{1}{\pi\left(R_2{}^2 - R_1{}^2\right)} \int_{R_1}^{R_2} (1 - \lambda \frac{r}{R_2}) \frac{F_0}{2\pi r L} 2\pi r dr \tag{12}$$

We find after integration:

$$I_{Mp} = \frac{F_0}{\pi L}\left(\frac{1}{R_2 + R_1} - \frac{\lambda}{2R_2}\right) \tag{13}$$

For example, with the following numeric values:

$$\begin{cases} F_0 = 36W, \quad \lambda = 0.1 \\ R_1 = 0.00785\, m, \quad R_2 = 0.2156\, m, \quad L = 0.8m. \end{cases}$$

We find

$I_{Mp} = 6.08 mW.cm^{-2}$

Case of a perfectly mixed flow

For a perfectly mixed flow and for the same diagram reactor (Figure 2), without absorption and mixture, but where UV radiation I_r and speed v_r depend on the *r* distance to the axis, it is possible to consider the UV radiation I_r in *r* varied as described in the equation (9). We will suppose at first the flow speed varies linearly between the center and the border according to a simple law of the same type:

$$v_r = \left(1 - \xi \frac{r}{R_2}\right) v_0 \tag{14}$$

Where, v_0 and ξ are the speed at the axis of reactor without a lamp and constant that characterizes the variation of speed as moving away from the axis of reactor.

We consider that, according to the mode of water insertion into the reactor, the speed in the center will be more important than the one in the border of reactor.

In this example, the speed in the periphery is lower than the speed in the center. In these conditions, we inscribe the fraction of the flow presented to a UV irradiation of the form (11) is equal to:

$$Q_r = \frac{1}{\pi\left(R_2{}^2 - R_1{}^2\right) v_m} \left(1 - \xi \frac{r}{R_2}\right) v_0 2\pi r dr \tag{15}$$

Where v_m is the average speed that can be related to the flow into the reactor, and its formulation is developed as follows:

$$v_m = \frac{1}{\pi\left(R_2{}^2 - R_1{}^2\right)} \int_{R_1}^{R_2} (1 - \xi \frac{r}{R_2}) v_0 2\pi r dr \tag{16}$$

Or:

$$v_m = v_0 \left(1 - 2\xi \frac{R_2{}^2 + R_1{}^2 + R_1 R_2}{3R_2\left(R_2 + R_1\right)}\right) \tag{17}$$

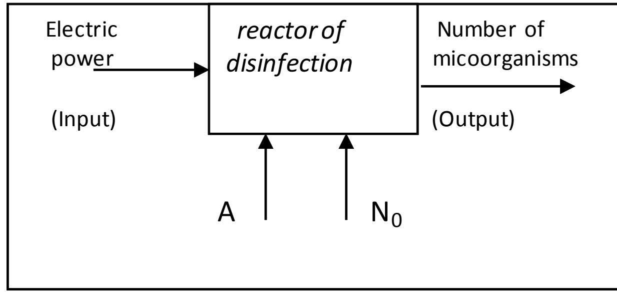

Figure 6. Block of the photoreactor in open buckle.

Therefore, the weighted average value *I* of UV radiation of rate of flow is written as:

$$I_{Mm} = \frac{1}{\pi\left(R_2{}^2 - R_1{}^2\right)v_m} \int_{R_1}^{R_2} (1-\lambda\frac{r}{R_2})\frac{F_0}{2\pi rL}(1-\xi\frac{r}{R_2})v_0 2\pi r dr \tag{18}$$

We find after integration:

$$I_{Mm} = \frac{F_0 v_0}{\pi L v_m}\left(\frac{1}{R_2+R_1} - \frac{\lambda+\xi}{2R_2} + \lambda\xi\frac{R_2{}^2+R_1{}^2+R_2R_1}{3R_2(R_1+R_2)}\right) \tag{19}$$

The weighted average value of UV radiation will be expressed as follows:

$$I_{Mm} = \frac{F_0 v_0}{\pi L(R_2+R_1)} \frac{1 - \frac{(\lambda+\xi)(R_1+R_2)}{2R_2} + \lambda\xi\frac{R_2{}^2+R_1{}^2+R_2R_1}{3R_2{}^2}}{1-2\xi\frac{R_2{}^2+R_1{}^2+R_2R_1}{3R_2(R_1+R_2)}} \tag{20}$$

Conditions and numeric values of the reactor are as follows:

$$\begin{cases} F_0 = 36W, \quad \lambda = 0.1 \\ \xi = 0.2, \quad R_1 = 0.00785\, m \\ R_2 = 0.2156\, m, \quad L = 0.8m \end{cases} \tag{21}$$

We find:

$$I_{Mm} = 6.3 \cdot mW.cm^{-2}$$

Formulation of the control problem

The objective of seeing control laws is to reduce the number of microorganisms at the exit of the UV reactor, while acting on the energizing illumination (*I*) of the UV rays at the entry of the reactor in a perfectly mixed flow (Figure 6).

The problem can be posed in the following way to give a variation of UV radiation intensity *I* in such a way the number *N* of microorganisms at the exit of the UV reactor is reduced according to the following constraints:

$$\begin{cases} \frac{N}{N_0} = A.\exp(-kIt) \\ I_{Mm} = \frac{F_0 v_0}{\pi L(R_2+R_1)} \frac{1 - \frac{(\lambda+\xi)(R_1+R_2)}{2R_2} + \lambda\xi\frac{R_2{}^2+R_1{}^2+R_2R_1}{3R_2{}^2}}{1-2\xi\frac{R_2{}^2+R_1{}^2+R_2R_1}{3R_2(R_1+R_2)}} \end{cases} \tag{22}$$

$$F_0 = 0.55P$$

and $P < P_{max}$

P_{max} =1.5 to 2.5 P_n (P_n=65W)

According to the numeric conditions of the system (22), the model (23) becomes:

$$\begin{cases} \frac{N}{N_0} = A.\exp(-kIt) \\ I = 1.75F_0 \\ F_0 = 0.55P \end{cases} \tag{23}$$

With $P < P_{max}$

For $y = N/N_0$, it is possible to express:

$$\begin{cases} \frac{dy}{dt} = -kIy \\ I = 1.75F_0 \\ F_0 = 0.55P \end{cases} \tag{24}$$

$$\text{Under} \begin{cases} P < P_{max} \\ y_0 = A \end{cases}$$

The parameters *k* and *A* are identified, using a constant intensity I = 6.3 mW.s.cm^{-2}, according to Figure 7.

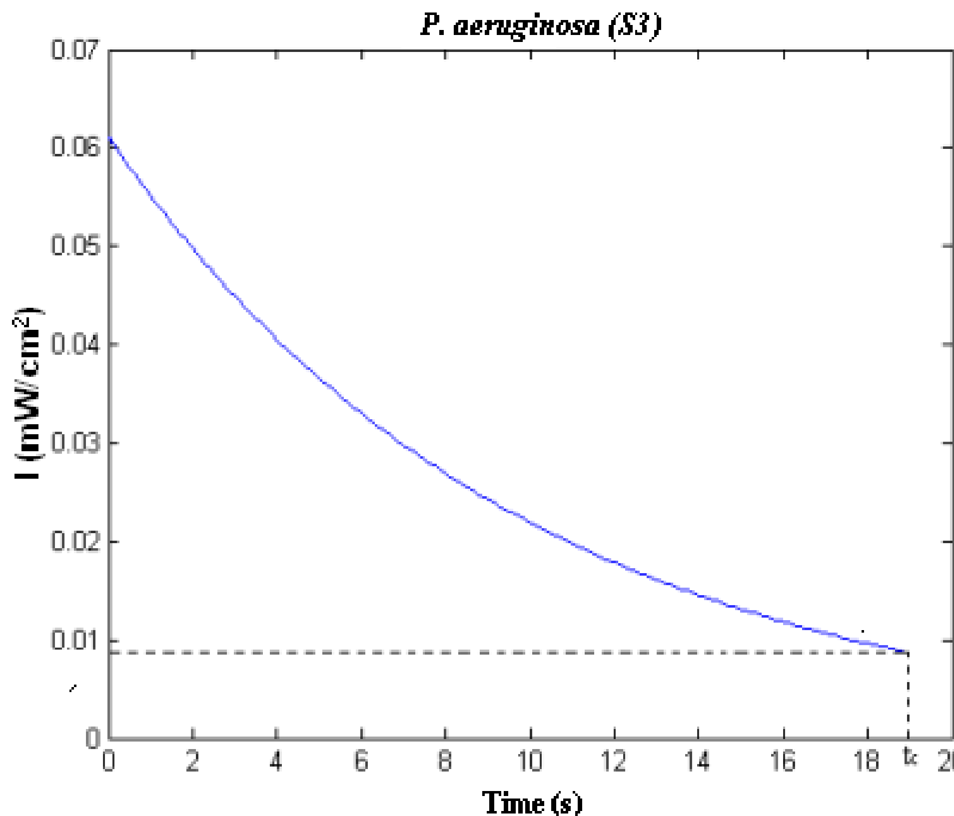

Figure 7. Variation of the *P. aeruginosa* (*S3*) inativation (fast dynamics) in open buckle.

The diversity of bacteria, and therefore the large number of models needed for all these bacteria, led us to work with the most resistant strain for UV radiation: *P. aeruginosa* ATCC 15442. The analysis and the optimization of results affirmed that such UV disinfection cannot be done only during the first kinetics (rapid) and it approves an important efficiency of UV inactivation (Brahmi and Hassen, 2011).

Where $y = N/N_0 = 0.0611exp(-0.0163It)$

Figure 8 gives a simulation of the model (25) for an average UV intensity I = 6.3 mW.s.cm⁻² and for the same UV dose used during the first dynamic of inactivation of *P. aeruginosa* ATCC 15442.

The duration of validity of the first dynamic (equation y_1), for the strain of *P. aeruginosa* and *for* I = 6.3 mW.s.cm⁻² as it is shown in Figure 9, is t_c = 19.04s. Now there is a commutation toward the second dynamic (equation y_2) which will take over. To improve result gained during the active interval, from instant zero to instant t_c, it allows a commutation to reach final value y_f towards the second dynamic that will be adjusted to the last value of $y_f = N_f/N_0$ gotten at $t = t_c$. The constant time delay of this kinetic response is τ *1* determined according to Figure 8 and it is equal to 9.7 s.

Conception of an improved control

Difficulties faced during the determination of relationships between the intensity of UV radiation, the arc tension and the arc current, in addition to the non emergence of a variable of control according to the Chick-Watson's model (Haas, 1990), made us to work with the flux emitted by the lamp and prompted us to find a way of improvement that considers the practical constraints of lamp functioning. Indeed, an increase in the UV dose and the UV radiation improves the results but leads to an increase of the lamp temperature and a risk of lamp damage; further, it will increase the losses in electric energy since the first instants of radiation are the most determinant of disinfection. Beyond this stage, the efficiency of the UV radiation decreases noticeably. This fact leads us to consider an exponential negative form (decreasing) for the energetic intensity I of the UV radiation:

$$I = I_0 e^{-\mu t} \quad (25)$$

Where: I_0 and μ are the initial value of the radiation previously fixed and depends on the maximum power of the lamp and the decreasing speed of the radiation that

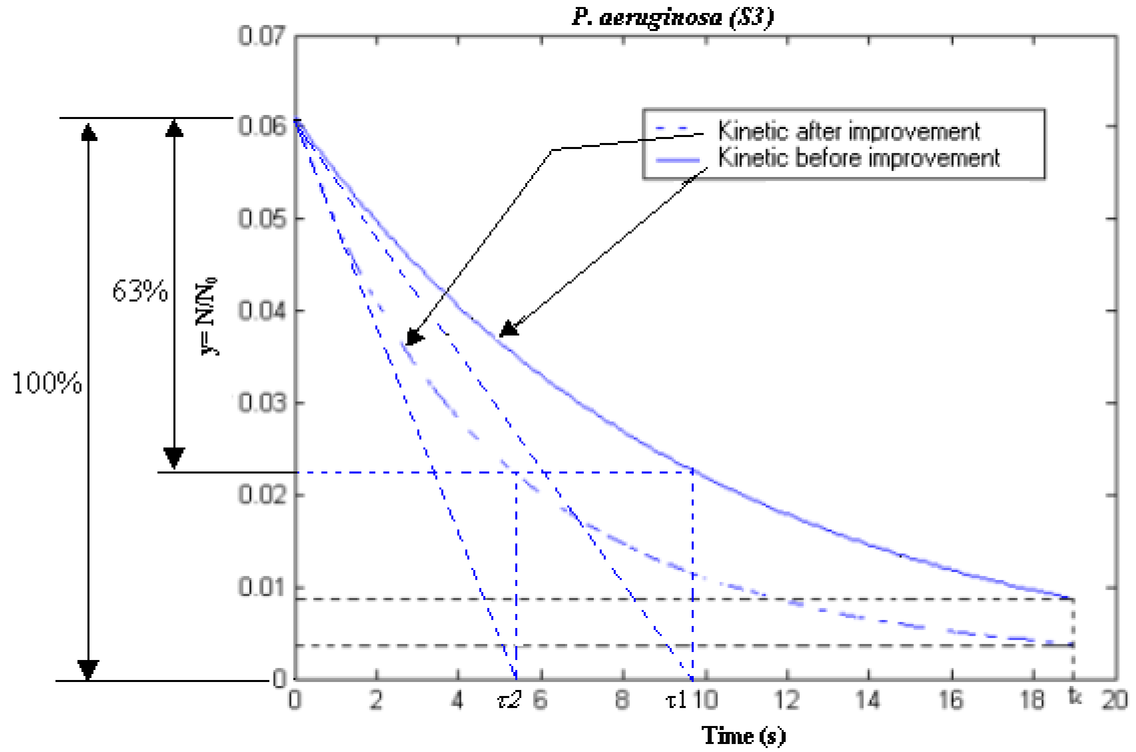

Figure 8. Comparison of two different variations of the energizing radiation on disinfection.

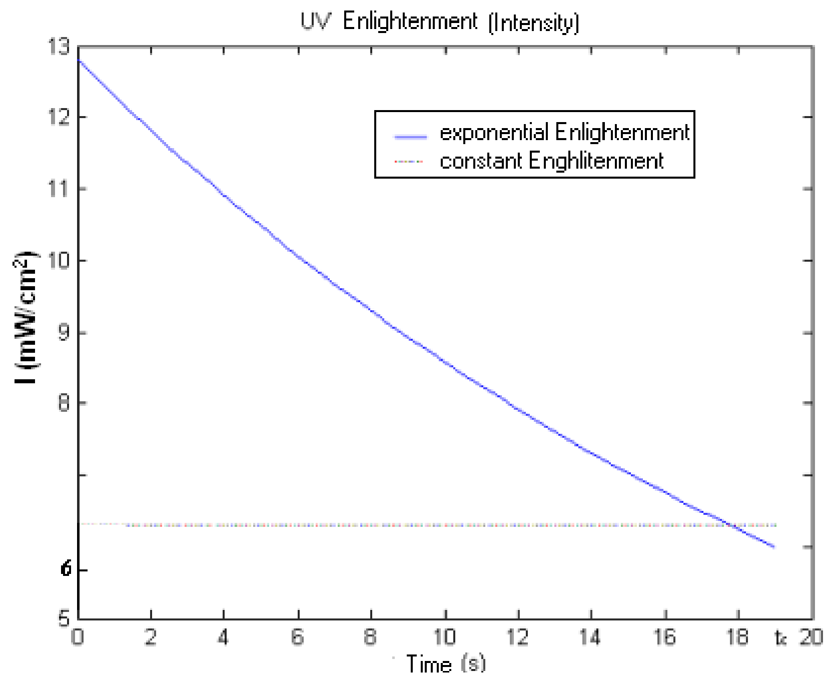

Figure 9. Comparison of variation of the UV intensity depending on the contact time.

can be also previously fixed, respectively. The choice and the dimensioning of these two factors (I_0 and μ) will be done according to the objectives to achieve.

In this condition of varying radiation (26), the UV flux F_0

emitted by the lamp becomes:

$$F_0 = \frac{I_0}{1.75}\exp(-\mu t) \quad (26)$$

After expressing electric power becomes:

$$P = \frac{I_0}{0.9625}\exp(-\mu t) \quad (27)$$

The model suggested is as follows:

$$\begin{cases} \frac{dy}{dt} = -kIy \\ \frac{dI}{dt} = -\mu I \end{cases} \quad (28)$$

Under these following initial and numeric conditions:

$$\begin{cases} y_0 = A = 0.0611 \\ I_0 = 12.8 \\ k = 0.0163 \\ \mu = 0.04 \end{cases} \quad (29)$$

We supposed in this situation the initial reduction *A* which is similar to a constant radiation intensity *I* to guarantee that if the results of disinfection are ameliorated in this precise case. We are sure that they will be improved for an initial radiation I_0 which is more interesting, and then an initial reduction which is more important.
For the strain *P. aeruginosa* ATCC 15442, the results of the model (28) under conditions (29), are given in Figure 8. After improvement of the kinetics of inactivation, the kinetics of disinfection accelerated with a constant time delay of $\tau 2$=5.35 s, and the number of bacteria at $t = tc$ became lower.

The simulation of the second equation of the model (29) is represented in Figure 8. The UV dose for a variation over time of the UV intensity (*I*) constitutes the surface placed between the axis of absciss as, the axis of ordinate and the curve of variation of intensity (*I*) over time. In this case, the dose for a fixed intensity (*I*) is 120 mW.s.cm²; on the other hand, in the case where the UV radiation would take a decreasing exponential variation of about 170.5 mW.s.cm^{-2}, there is an increase of about 42%. The variation of the electric power consumed for the model (27) under conditions (29) is given in Figure 9.

A negative exponential variation of the instantaneous electric power and then a decreasing exponential variation of the average energetic radiation for a perfectly mixed flowing (Figure 10), improved the result of disinfection similar to 49%; this is equivalent to 10^6 cells of *P. aeruginosa* at the entry of the reactor, with an improvement of 4900 inactivated cells after the application of the new variation of the radiation; in parallel, the kinetics of disinfection is suitably improved.

Conclusion

The modeling study of UV disinfection kinetics for all studied strains showed that the law of disinfection proposed by the model of Chick-Watson poorly simulated the experimental data. A divergence occurred on the rate of inactivation that was not quite linear. Thus, the original form of this model is not representative of disinfection kinetics.

Modification taking into account the change of disinfection rate during the process did not significantly improved results, indeed. The application of a first order law to the kinetics model of disinfection was therefore possible, if we assumed the existence of two successive steps of different kinetics. The adjustment of the same model but considering an initial reduction describes quite well the kinetics of disinfection for the most studied strains.

The development and improvement of water disinfection remain an urgent goal. From describing the difficulties and the complexities of models of low pressure discharge lamps used in UV water disinfection and from determining an average UV radiation for ideal water flowing, we aimed to freeze a curve of variation throughout time of the average UV intensity. Investigations were done in the optimal conditions of functioning using the most UV resistant bacterial strain as *P. aeruginosa* ATCC 15442 and the UV flux emitted represents 55% of the electric power consumed. Results are satisfactory in considering only the theoretical aspect of UV pathogenic bacteria inactivation.

The complexities of different modeling factors and parameters of UV discharge lamps, the absence of mathematical relations between voltage and fluxes emitted will remain interesting subjects for future research.

Conflict of interest

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

This study was supported by the European Union (Avicenna Project n° 93AVI054). We express great thanks to all the team of the laboratory of Water Treatment and Recycling, Borj-Cedria Techno-park that contributed from near and far to the success of this work, particularly Mr. Aissaoui and Ms Mabrouk for their help.

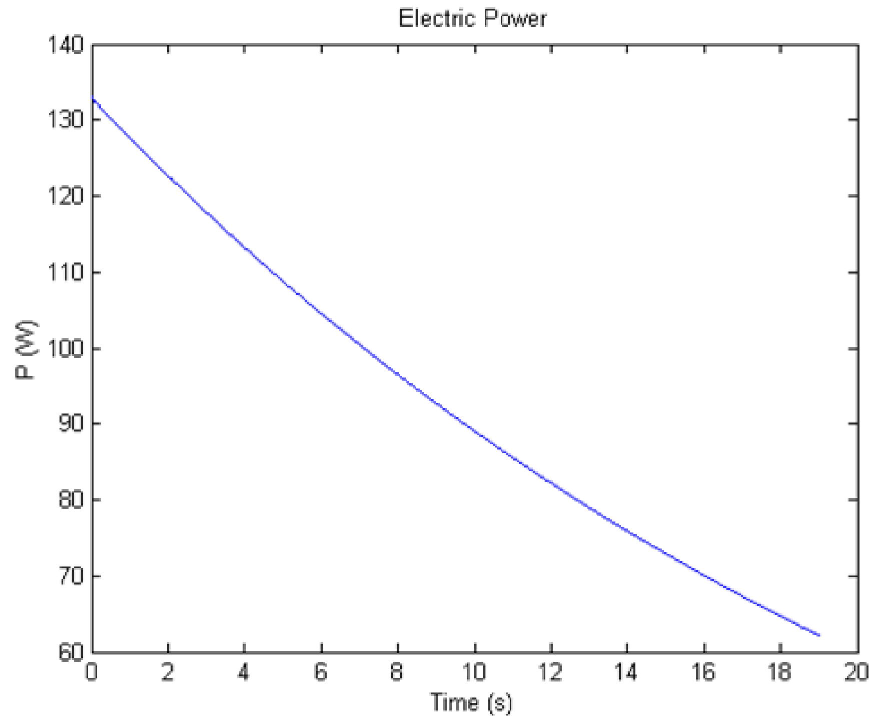

Figure 10. Variation of the electric power consumed.

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Vol. 8(15), pp. 1601-1609, 9 April, 2014
DOI: 10.5897/AJMR2014.6720
Article Number: F4BCC3D43824
ISSN 1996-0808
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http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Growth performance, carcass characteristics and meat quality of Hanwoo steers fed fermented liquid whey inoculated with lactic acid bacteria

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Received 18 February, 2014; Accepted 17 March, 2014

The present study was conducted to evaluate the effects of liquid whey fermented with lactic acid bacteria on the growth performance, carcass characteristics, meat composition, fatty acid profile and meat oxidative stability of Korean Hanwoo steers. Twenty-four homogenous Hanwoo steers (22-months-old) were randomly distributed into two dietary treatments with four replications of three steers per treatments. Basal diet was supplemented with 0.2% fermented liquid whey (FLW) and its effects were compared with those of a control. At the end of feeding trial, steers were slaughtered and graded for quality and yield factors by a trained carcass evaluator. Overall, dietary supplementation with 0.2% FLW increased the body weight, average daily gain and gain to feed ratio (P<0.05) of Hanwoo steers. None of the carcass characteristics were affected by dietary FLW. The ether extract and ash contents of loin eye meat were reduced, whereas the calcium and iron contents were increased in response to dietary FLW supplementation (P<0.05). At fresh state, the malondialdihyde (MDA) value of meat was reduced in response to FLW supplementation (P=0.003), whereas no difference was observed at week 1. During weeks 2 and 3, dietary FLW tended to reduce the MDA value of loin eye meat (P<0.10). Dietary supplementation with FLW increased the concentration of linoleic acid, sum of polyunsaturated fatty acids (PUFAs) and n-6 PUFAs (P<0.05). Overall, supplementation of diet with 0.2% FLW exerted beneficial effects on growth performance, meat composition and meat oxidative stability without affecting carcass characteristics, indicating that it can be used as a feed additive in finishing beef cattle.

Key words: Liquid whey, lactic acid bacteria, growth performance, meat composition, oxidative stability, Hanwoo steers.

INTRODUCTION

The potential removal of antibiotic growth promoters (AGPs) from the beef cattle industry has created renewed interest in the use of probiotics. Lactic acid bacteria (LAB), which are the most common types of microorganisms used as probiotics in animal nutrition, are mainly administered after freeze-drying in the form of tablets, paste or powder directly or by mixing with feed. An alternative to delivering large numbers of LABs is the use of fermented feed, in which LABs are present as viable cells with metabolites produced during the fermentation process (Amezcua et al., 2007). A number of scientific studies have demonstrated that fermentation with LAB occurs more rapidly, with greater control and less production of undesirable fermentation products (Demecková et al., 2002; Lawlor et al., 2002); therefore, it is considered a biosafe method for fermenting animal feed

(Kobashi et al., 2008). Liquid feeds fermented with specific strains of LAB exhibit probiotic properties and can tolerate the acidic conditions in the stomach and bile acids in the small intestine (Geary et al., 1999; Alvarez-Olmos and Oberhelman, 2001). Therefore, fermented liquid feeding is considered to be an alternative to anti-microbial growth promoters.

Utilization of liquid feeds in animals has created an opportunity for recycling of liquid co-products from the human food industry. Whey, a valuable co-product of cheese, curds and casein, is a slightly acidic, yellow-green liquid that remains after the coagulation of milk by rennet or by the reduction of its pH (Green, 1977). Liquid whey is composed of lactose (5%), water (93%), proteins (0.85%), minerals (0.53%), a minimum amount of fat (0.36%), and some non-protein nitrogen (Pescuma et al., 2008). The primary whey proteins are β-lactoglobulin (58%) and α-lactalbumin (13%), whereas immunoglobulin, serum albumins and protease peptones are present to a lesser extent (Pescuma et al., 2008) and afford a number of beneficial effects. Whey protein can improve protein synthesis and mineral absorption, whereas reducing blood sugars and blood lipids and improving insulin sensitivity (Pal et al., 2010; Pilvi et al., 2007). Considerable amount of whey is disposed of as waste, causing serious environmental problems. Owing to the nutritional value of whey, significant efforts have been made over the past few years to identify new outlets for whey utilization and reduce environmental pollution. Among the proposed solutions, the utilization of whey as animal feed is by far the most promising. For ruminants, whey is mainly used as a silage additive to improve the quality of low grade forage or agro-industrial by-products. It is evident that adult ruminants are able to use whey or derived products more efficiently than poultry, pigs or rats (Schingoethe, 1976).

Fermentation of whey by LAB has been shown to result in the production of organic acids, mainly lactic acid (Weiberg, 2003), as well as other metabolites such as aroma compounds that contribute to the flavor and texture of fermented feed (Mauriello et al., 2001). The production of ammonium lactates as a source of crude protein (defined as total N × 6.25) for cattle by fermentation of cheese whey have been described by Reddy et al. (1976). The proteolytic enzymes of the lactic acid bacteria contributed in the formation of amino acids and vitamins in fermented whey (Law and Haandrikman, 1997) and thereby may increase the availability of amino acid for rumen microorganism. The fermented whey had its lactose, which could also serve as an energy source for rumen microorganisms. In addition, LABs itself can interact with rumen microorganisms in such a way that their activity is enhanced and fiber degradability is improved (Weinberg, 2003). A limited number of animal experiments have been conducted using fermented liquid whey as feed additives (Amezcua et al., 2007; de Oliveira et al., 2012). This study was aimed to investigate the effects of fermented liquid whey (FLW) on the growth performance, carcass quality, meat composition and fatty acid profile and meat oxidative stability of finishing Hanwoo (Korean native cattle) cattle.

MATERIALS AND METHODS

Fermentation of whey by lactic acid bacteria

A commercially available freeze-dried probiotic starter culture, YO-MIX^{TM} 211, was used for the fermentation of whey (Danisco Culture Co., Denmark). YO-MIX^{TM} 211 contains a mixture of *Streptococcus thermophilus*, *Lactobacillus delbreuckii* subspecies *bulgaricus*, *Lactobacillus acidophilus* and *Bifidobacterium lactis.* The cheese whey was obtained from the Dairy Microbiology Laboratory of the Department of Animal Science and Technology, Sunchon National University, Korea. Before fermentation, the whey was sterilized at 63°C for 30 min, after which it was cooled to 37°C using a water bath. After cooling, 1.5% YO-MIX^{TM} 211 was added to the whey and was fermented for 3 to 4 h at 37°C and pH 4.7 to 4.8. After fermentation, the fermented product was stored in the refrigerator until added to the feed. The fermented liquid whey (FLW) contained approximately 1 × 10^8 cfu/mL lactic acid bacteria, 6.5% dry matter, 19.2% crude protein, 0.12% ether extract and 27.21% SNF.

Experimental design, animals and diets

A total of 24 homogenous Hanwoo steers (22-months-old; 599.58 ± 19.39 kg body weight) were randomly distributed into two dietary treatments with twelve steers per treatments in a completely randomized design. A group of three steers was considered as one replication. Experimental diets consisted of a control (basal diet) and a diet supplemented with 0.2% FLW. The feeding trial was carried out at the Sunchon National University experimental farm and continued for 6 month (up to 26 month of age). The steers were housed individually in well ventilated clean shed having individual feeding and watering arrangements. A commercially available total mixed ration (TMR) feed was used as the basal diet (Table 1). The experimental feed was supplied twice per day in the morning and evening at a rate of 10.87 kg/steer/day on DM basis. The fermented whey was sprayed directly onto the basal diet before each feeding period. Water was available *ad libitum*. Lighting and other management methods were carried out in accordance with general practice. All experimental procedures used in this study were approved by the Animal Care and Use Committee of Sunchon National University.

Samples of the basal and FLW diet were ground through a 1 mm

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Abbreviations: FLW, Fermented liquid whey; **LAB,** lactic acid bacteria; **MDA,** malondialdihyde, **MUFA,** monounsaturated fatty acid; **PUFA,** polyunsaturated fatty acid; **SFA,** saturated fatty acid; **TBARS,** thiobarbituric acid reactive substance.

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Table 1. Feed ingredients and chemical composition of TMR diet.

Item	Control	FLW 0.2%
Ingredient (%, DM basis)		
Corn grain	43.33	43.33
Wheat	10.50	10.50
Tapioca	3.45	3.45
Corn gluten feed	11.78	11.78
Wheat flour	2.00	2.00
Soybean meal	0.50	0.50
Rapeseed meal	2.00	2.00
Coconut meal	8.23	8.23
Palm kernel expeller	9.00	9.00
Lupin	6.00	6.00
Molasses	3.00	2.80
Liquid fermented whey	0.00	0.20
Vitamin mineral premix[a]	0.21	0.21
Analysed chemical composition		
Dry matter (% of natural matter)	83.26	78.56
Crude protein (% DM)	12.62	15.24
Ether extract (% DM)	3.63	6.52
Crude fiber (% DM)	16.68	17.58
Crude ash (% DM)	5.48	5.83
Calcium (% DM)	0.82	1.10
Phosphorus (% DM)	0.38	0.50
NDF(% DM)	21.70	22.15
ADF (% DM)	10.70	10.25
Calculated composition (Mcal/kg DM)		
Net energy for maintenance (NEm)	1.99	2.01
Net energy for gain (NEg)	1.34	1.35

[a]Premix provided the following nutrients per kg of diet: Vitamin (Vit) A, 9,000,000 IU; Vit. D_3, 2,100,000 IU; Vit. E, 15,000 IU; Vit. K, 2,000 mg; Vit. B_1, 1,500 mg; Vit. B_2, 4,000 mg; Vit. B_6, 3,000 mg; Vit. B_{12}, 15 mg; Pan-Acid-Ca, 8500 mg; Niacin, 20,000 mg; Biotin, 110 mg; Folic-Acid, 600 mg; Co, 300 mg; Cu, 3,500 mg; Mn, 55,000 mg; Zn, 40,000 mg; I, 600 mg; Se, 130 mg.

screen to analyze the chemical composition. Crude protein (988.05), ether extract (991.36), crude ash (942.05) and crude fibre (962.09) contents were analyzed according to procedures from the Association of official Analytical Chemists (AOAC, 2000). Calcium and phosphorus were analyzed using an Atomic Absorption Spectrophotometer (AA-6200, Korea). The neutral detergent fiber (NDF) and Acid Detergent Fiber (ADF) were determined according to Van Soest et al. (1991). The ADF values were used to predict DE (digestible energy) values of the ration using the equation TDN% = 88.936 – 0.653 ADF (for TMRs and miscellaneous mixed feeds) and 1 kg TDN = 4.4 Mcal DE (NRC, 1996). The calculated DE values of the feed were used to predict the net energy for maintenance (NEm) and net energy for gain (NEg) of the diet using calculated metabolizable energy (ME = DE × 0.82) values according to the following formula (NRC 1996):

$$NEm = 1.37ME - 0.138\ ME^2 + 0.0105ME^3 - 1.12$$

$$NEg = 1.42ME - 0.174\ ME^2 + 0.0122ME^3 - 1.65$$

Measurements and analyses

Body weights were taken before feeding and watering using a platform scale at 3-month intervals from the onset of the experiment until the end. Feed bunks were cleaned and residues were collected daily and weighed at intervals corresponding to weigh dates throughout the trial. The feed intake was calculated on DM basis. Feed efficiency (gain/feed) was calculated as the ratio between average body weight gain and feed intake on DM basis.

All steers were slaughtered in a commercial packing facility of Suncheon City and graded for quality and yield factors by a trained carcass evaluator. Grading of carcasses was carried out in accordance with Korean beef carcass grading standards (KAPE, 2012). Each beef carcass was assigned one of five quality grades (1++, 1+, 1, 2 or 3). Grades were primarily based on marbling score and additionally adjusted according to meat color, fat color, texture of lean meat and maturity. One of three yield grades (A, B or C) was determined by assessing the live weight, carcass weight, back fat thickness and longissimus muscle area.

Table 2. Effect of dietary supplementation with fermented liquid whey (FLW) on growth performance of Hanwoo steers from 22 to 28 months of age.

Item	Treatment		SEM	P-value
	Control	FLW 0.2%		
Initial body weight (kg)	603.42	595.75	19.39	0.79
Final body weight (kg)	764.50[b]	818.00[a]	13.81	0.04
Average daily gain (kg/day)	0.90[b]	1.24[a]	0.04	0.001
Average daily feed intake of DM (kg/day)	10.83	10.83	0.07	0.98
Feed efficiency (gain/feed)	0.08[b]	0.11[a]	0.01	0.001

For each treatment, data are presented as the mean value of four replicate groups with three steers per replication (n = 12). Within a row, means without a common letter differ significantly (P<0.05).

To investigate the meat composition, loin eye meats from selected Hanwoo steers were excised and ground with a meat grinder. The moisture (934.01), crude protein (988.05), ether extract (991.36) and crude ash (942.05) contents of the samples were then determined using the methods described by AOAC (2000). The calcium (Ca), iron (Fe) and magnesium (Mg) contents of carcasses were determined using an atomic absorption spectrophotometer (AA-6200, Korea).

To determine the cholesterol content, 1 g of each meat sample was mixed with reference material (100 µg of 5 α-cholesterol) and homogenized with 0.5 N KOH (aq) and 22 mL of ethanol, after which it was subjected to saponification at 23°C for 6 h. The total cholesterol was subsequently extracted with hexane and analyzed by gas chromatography (DS 6200, Donam Co., Seongnam, Gyeonggi-do, Korea) using a gas chromatograph equipped with a flame ionization detector and a Hewlett Packard HP-5 capillary column (J&W Scientific, USA) 30 m in length with a 0.32 mm internal diameter and 0.25 µm polyethylene glycol-film thickness. Nitrogen was used as the carrier gas. The initial oven temperature was held at 250°C for 2 min, increased by 15°C/min to 290°C (held for 10 min), and then by 10°C/min to a final temperature of 310°C (held for 10 min). The other chromatographic conditions were as follows: injector and detector temperatures, 280°C; split ratio, 50:1; sample volume injected, 2 µL. Cholesterol content was expressed as mg/100g meat.

Meat fatty acids were determined by the methyl ester extraction methods according to Yang et al. (2003) and analyzed by gas chromatography using a DS 6200 gas chromatograph (described above) equipped with a flame ionization detector and a Hewlett Packard HP-5 capillary column (J&W Scientific, USA) 30 m in length with a 0.32 mm internal diameter and 0.25 µm polyethylene glycol-film thickness. Samples were injected by an auto-sampler. During analysis, the injector temperature was maintained at 250°C and the detector temperature was maintained at 270°C. The initial oven temperature was held at 140°C for 1 min, after which was increased by 10°C/min to 220°C, which was held for 2 min, then further increased by 2°C/min to 240°C, which was held for 9 min. Nitrogen was applied at 1.0 mL/min as the carrier gas and hydrogen was applied at 30 mL/min as the makeup gas. Individual fatty acids were identified by comparing their retention times with those of an authenticated standard fatty acid mix (Supelco 37; Sigma Chemical Co. Ltd., Poole, UK). Individual fatty acids were corrected by their relative response factors using the value of the internal standard and expressed as g/100 g of total fatty acids identified. Fatty acids were grouped as saturated (SFA), monounsaturated (MUFA) or polyunsaturated (PUFA), and the ratios of PUFA:SFA and n-6/n-3 were calculated.

To determine the oxidative stability, meat samples were preserved in a refrigerator at 4.5°C and the thiobarbituric acid reactive substance (TBARS) values were assayed when fresh as well as at 1, 2 and 3 weeks according to the method described by Sarker and Yang (2011). Color value was determined using a Vis-Spectrophotometer (Model 20D+, Milton Roy, USA) based on the absorbance at 530 nm, and TBARS values are expressed as micromoles of malondialdehyde (MDA) per 100 g of meat sample.

Statistical analysis

All data were analyzed as a completely randomized design using the GLM procedure of the Statistical Analysis System (version 9.1; SAS Ins. Inc., Cary, NC, USA, 2003) based on the statistical model:

$$Y_{ij} = \mu + T_i + e_{ij}$$

Where Yij = dependent variable of the jth animal on the ith treatment; µ = overall mean; Ti = the fixed effect of ith treatment effect (I = 1, 2); Eij = random residual (error) associated with the dependent variable from the jth animal on the ith treatment.

A group of three steers served as the experimental unit for all parameters. Model included the effects of diets. Variability in data was expressed as the standard error of the means (SEM) and Duncan's multiple range tests were used to examine significant differences among treatment means. A P≤0.05 was considered to indicate statistical significance, whereas 0.5<P≤0.10 was considered to indicate trends.

RESULTS

There was no differences in the initial body weight of the steers (P>0.79) between dietary treatments (Table 2). Steers fed 0.2% FLW supplemented diet showed higher final body weight (P<0.04) and average daily gain relative to the control (P=0.001). Steers in both treatment groups had similar feed intakes; however, the gain/feed ratio was higher in the 0.2% FLW supplemented group (P=0.001). None of the measured carcass characteristics were affected by dietary supplementation with FLW (Table 3).

The effects of FLW on meat composition, cholesterol and trace mineral content of Hanwoo carcasses are shown in Table 4. Moisture and crude protein contents of loin meat were unaffected by FLW dietary treatment (P>0.97); however, ether extract (P<0.05) and ash (P<0.04) contents were reduced in the loin eye meat of steers fed the diet supplemented with 0.2% FLW. There was no difference among treatments in the cholesterol

Table 3. Effects of dietary supplementation with fermented liquid whey (FLW) on carcass characteristics of Hanwoo steers.

Item	Treatment		SEM	P-value
	Control	FLW 0.2%		
Live weight (kg)	764.50	818.00	21.10	0.12
Hot carcass weight (kg)	432.50	463.00	14.07	0.18
Dressing percent (%)	56.55	56.59	0.37	0.95
Carcass yield grade	3.00	2.75	0.28	0.58
Carcass quality grade	1.75	1.25	0.20	0.13

The carcass yields are graded as 3, grade A; 2, grade B; and 1, grade C. The carcass quality are graded as 5 (1++), 4 (1+), 3 (1), 2 (2), and 1 (3) (According to KAPE, 2012). Data are presented as the mean value of four replicate groups with three steers per replication (n = 12).

Table 4. Effects of dietary supplementation with fermented liquid whey (FLW) on meat composition, cholesterol and trace mineral content of Hanwoo carcasses.

Item	Treatment		SEM	*P*-value
	Control	FLW 0.2%		
Moisture (%)	68.44	69.92	0.73	0.21
Crude protein (%)	22.12	22.16	0.64	0.97
Ether extract (%)	8.3[a]	7.00[b]	0.36	0.05
Crude ash (%)	1.11[a]	0.93[b]	0.05	0.04
Cholesterol (mg/100 g meat)	25.09	27.06	1.36	0.36
Ca (mg/100 g)	0.25[b]	0.35[a]	0.02	0.02
Fe (mg/100 g)	0.40[b]	0.49[a]	0.02	0.05
Mg (mg/100 g)	2.39	2.20	0.08	0.21

Data are presented as the mean value of four replicate groups with three steers per replication (n = 12). Within a row, means without a common letter differ significantly (P<0.05).

content of meat (P>0.36). Dietary supplementation with 0.2% FLW increased the Ca (P<0.02) and Fe (P<0.05) contents of loin meat, whereas the Mg content remained unaffected (P>0.21).

The effects of dietary FLW on the fatty acid composition of Hanwoo steer loin eye meat are presented in Table 5. Dietary supplementation of FLW increased the linoleic acid concentration (P<0.03) of the loin eye meat of Hanwoo steers. The sum of PUFAs (P<0.04) and n-6 PUFAs (P<0.05) were also elevated in response to FLW supplementation.

In the fresh state, the MDA value was lower in the FLW-supplemented group than in the control (P=0.003; Figure 1), whereas it did not differ from the control group during week 1 (P>0.33). During weeks 2 and 3, a reducing tendency (P<0.10) was found in the MDA value of loin meat obtained from the FLW-supplemented group.

DISCUSSION

Utilization of whey in feed provided to ruminants constitutes one of the newest and most rapidly exploitable means of application because it is inexpensive, easy to put into practice and offers a good method of utilizing non-protein nitrogen sources for ruminants. Whey fermented by LAB can improve ruminant performance by synchronizing rumen fermentation, being a source of organic acid and living bacteria (de Oliveira et al., 2012). The results showed significant improvements in body weight, average daily gain and feed efficiency (gain/feed) in steers fed 0.2% FLW diets versus the control. Manipulation of ruminal fermentation has been one of the methods used to increase ruminant productivity (de Oliveira et al., 2012). The low protein and high rapidly degradable carbohydrate (lactose) content of whey led to the assumption that this by-product would help synchronize rumen fermentation, thereby improving performance. Fermentation of whey with LAB produces a considerable amount of lactic acid through metabolism of whey lactose. Dietary addition of fermented whey can continue the lactose fermentation process, as well as that of other carbohydrates that reduce the pH of the rumen and stimulate the growth of lactate utilizing bacteria (Krehbiel et al., 2003). Once lactate utilizing bacteria concentrations increase, the ability to metabolize lactate

Table 5. Effects of dietary supplementation with fermented liquid whey (FLW) on meat fatty acid profile of Hanwoo steers.

Fatty acid (% of total fatty acid)	Treatment		SEM	P-value
	Control	ACP 0.5%		
Myristic acid (C14:0)	4.50	4.17	0.45	0.62
Palmitic acid (C16:0)	31.52	32.96	2.09	0.69
Stearic acid (C18:0)	0.05	0.02	0.02	0.27
Palmitoleic acid (C16:1n-7)	3.73	4.42	0.90	0.67
Oleic acid (C18:1n-9)	48.84	45.74	3.15	0.58
Linoleic acid (C18:2n-6)	9.27[b]	10.51[a]	0.30	0.03
Linolenic acid (C18:3n-6)	0.51	0.48	0.06	0.82
α-Linolenic acid (C18:3n-3)	0.09	0.10	0.01	0.77
Eicosenoic acid (C20:1n-9)	0.15	0.13	0.02	0.48
Arachidonic acid (C20:4n-6)	0.62	0.72	0.16	0.72
Eicosapentaenoic acid (C20:5n-3)	0.34	0.35	0.03	0.70
Adrenic acid (C22:4n-6)	0.27	0.25	0.04	0.86
Docosahexaenoic acid (C22:6n-3)	0.14	0.17	0.03	0.58
∑ Saturated fatty acid (SFA)	39.75	41.54	3.04	0.73
∑ Monounsaturated fatty acid (MUFA)	49.04	45.88	3.13	0.57
∑ Poly unsaturated fatty acids (PUFA)	11.22[b]	12.58[a]	0.36	0.04
∑ Unsaturated fatty acids (UFA)	60.25	58.46	3.04	0.73
∑n-3 PUFA	0.56	0.62	0.03	0.30
∑n-6 PUFA	10.66[b]	11.96[a]	0.35	0.05
MUFA/SFA	1.24	1.19	0.15	0.82
PUFA/SFA	0.29	0.31	0.02	0.45
UFA/SFA	1.52	1.50	0.17	0.92
n-6/n-3	19.24	19.71	1.39	0.82

Data are presented as the mean value of four replicate groups with three steers per replication (n = 12). Within a row, means without a common letter differ significantly (P<0.05).

to volatile fatty acid (primarily propionic acid) also increases (Counotte et al., 1983). If propionic acid production is both energetically enhanced and proportionately increased, then it is likely that the energy available to the animal also increases, thereby increasing growth performance. Another possibility is that LABs produce bacteriocins in the FLW, which might inhibit detrimental microorganisms (*Escherichia coli*) in the rumen, resulting in improved fiber digestibility (Ramaswami et al., 2005). The beneficial effects of LABs to improve feed efficiency and daily gain of feedlot cattle were also reported by Swinney-Floyd et al. (1999) and Galyean et al. (2000), however no differences were reported for carcass characteristics. Krehbiel et al. (2003) also reviewed that, LAB did not affect the dressing percentage, USDA yield grade, USDA quality grade and marbling score of beef cattle, which is consistent with our findings.

Ether extract refers to the crude mixture of fat-soluble material present in a sample and includes glycerides (mono-, di-, tri-), phospholipids, steroids, free fatty acids, fat soluble vitamins, and cholesterol. Stimulation of the lipolytic activity in adipose tissue or inhibition of fat synthesis is one method of reducing ether extract content in animal tissue. A number of scientific studies have confirmed the lipolytic activity of LABs through the production of lipases and esterases (Meyers et al., 1996). According to Medina et al. (2004), LABs can hydrolyze triglycerides, releasing most short and medium chain, and essential fatty acids, which are valuable to health conscious consumers. LABs also exhibit anti-lipogenic activity to inhibit fat synthesis by reducing the expression of sterol regulatory element-binding proteins (SREBP), which controls the lipid composition of animal cells (Sakai et al., 1998). Yonejima et al. (2013) reported that the administration of *Lactobacillus* fermented soymilk and soy yogurt reduced the expression of SREBP and hepatic lipogenesis in rats. Furthermore, whey is reported to contain substantial amount of branched-chain amino acid, leucine, which is able to promote muscle protein synthesis, instead of fat storage in the adipose tissue (Pilvi et al., 2007). In the present study, the reduction in ether extract contents in the loin eye meat of Hanwoo steers was likely due to the synergistic effects of whey protein and LABs.

Dietary supplementation of FLW increased the Ca and

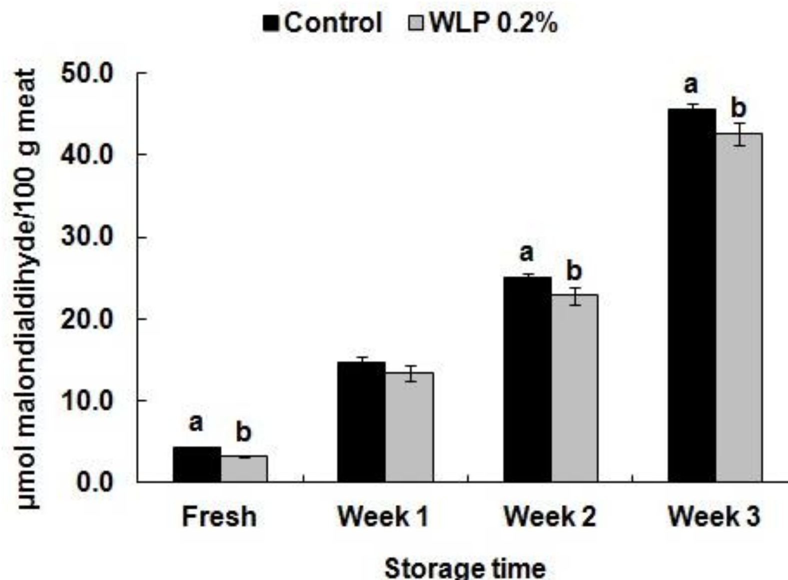

Figure 1. Effects of dietary fermented liquid whey (FLW) on thiobarbituric acid reactive substance (TBARS) values in loin eye meats of Hanwoo beef. TBARS values are expressed as micromoles of malondialdehyde (MDA) per 100 g of meat. Data are presented as the mean ± S.E. Bars at a particular time point without a common letter indicate a significant difference (P<0.05).

Fe content of beef. Whey itself is a good source of calcium, magnesium, phosphorus, iron and zinc with high purity and bioavailability. Fermentation of whey by LABs produces lactic acid by using lactose, which reduces the pH of the rumen and the digesta pH in the lower tract and therefore may stimulate dietary Ca and Fe absorption by slowing the gastric emptying rate (Chonan et al., 1998). Low pH also increases the buffering capacity, which keeps the Fe in a bioavailable form (Hallberg and Rossander, 1982).

The consumption quality of meat largely depends on its fatty acid composition (Wood et al., 1999). Consumers are increasingly demanding products with higher PUFA content because of their beneficial effects in preventing cardiovascular disease (Ander et al., 2003). In this experiment, dietary supplementation with FLW signify-cantly increased the proportion of linoleic acid, total PUFAs and total n-6 PUFAs in Hanwoo beef without affecting the SFA, and the proportion of n-6/n-3 PUFA indicates its positive effects on meat quality. A previous study conducted by Ross et al. (2012) reported that dietary supplementation of *Lactobacillus amylovorus* and *Enterococcus faecium* can increase the concentration of linoleic acid and total PUFAs in pig muscles. One possible explanation for this is that LABs may reduce the oxidation of PUFAs by scavenging free radicals (Virtanen et al., 2007) or degrading the superoxide anion and hydrogen peroxide (Kullisaar et al., 2002).

Malondialdehyde, a product of lipid oxidation, is determined by the TBARS test as an estimate of the development of rancidity in meat products. The rate of lipid oxidation can be effectively retarded by the use of antioxidants, and the antioxidative potential of LAB has been previously reported (Virtanen et al., 2007). Lactic acid bacteria strains exhibit antioxidative activity in varying ways, which makes it very difficult to identify individual mechanisms or compounds responsible for its antioxidative activity. Kaizu et al. (1993) reported that LABs are able to decrease the risk of accumulation of reactive oxygen species (ROS). Furthermore, Korpela et al. (1997) and Kullisaar et al. (2002) reported the superoxide anion and hydrogen peroxide degradation capacity of LABs. In addition, several studies have described the antioxidant potential of whey protein (Chen et al., 2003; Virtanen et al., 2007). Previous studies conducted by Osuntoki and Korie (2010) revealed the development of antioxidant activity in fermented milk whey inoculated with *Lactobacillus* species. Virtanen et al. (2007) reported that milk fermented with mixed cultures of LAB resulted in higher radical scavenging activity than milk fermented with single bacterial strains. In this study, dietary supplementation of FLW significantly reduced the MDA value of Hanwoo beef, which was ascribed to a synergistic effect of whey protein and LABs.

In conclusion, dietary supplementation with 0.2% fermented liquid whey (FLW) can improve the growth performance and meat composition of finishing Hanwoo steers without affecting the carcass characteristics. In addition, feeding on FLW was also found to be beneficial to improve meat storage quality by reducing the oxidative

rancidity of beef. Therefore, these results suggest FLW at 0.2% level as a functional feed additive for finishing beef cattle. Further investigation with different dose level is suggested to justify the results.

Conflict of interests

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

ACKNOWLEDGEMENTS

The authors are grateful to the Rural Development Administration (Project No. PJ007789) and Korea Institute of Planning and Evaluation for Technology of Agriculture, Forestry, Fisheries and Food (Project No. ARPC108123-3), Republic of Korea. The authors also thank Professor Inhyu Bae for proving the cheese whey for this experiment.

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Vol. 8(15), pp. 1645-1655, 9 April, 2014
DOI: 10.5897/AJMR2013.6598
Article Number: A82EDBC43860
ISSN 1996-0808
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http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Isolation of *Lactobacillus* strains with probiotic potential from camel's milk

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Received 31 December, 2013; Accepted 17 March, 2014

The study aimed to isolate and evaluate the probiotic potential of *Lactobacillus* species from fresh and fermented camel's milk samples. Isolates were identified morphologically and biochemically. Biochemical features investigated included temperatures, pH and NaCl concentrations effects on growth, survival in simulated gastrointestinal tract conditions, bacteriocin like activity and antibiotic resistance of the tested strains. Thirty four (34) isolates coded M1 to M 34 belonging to different *Lactobacillus* species (41% *Lactobacillus paracasei* ssp. *paracasei*, 23% *Lactobacills plantarum*, 18% *Lactobacillus rhamnosus*, 12% *Lactobacillus fermentum*, 6% *Lactobacillus brevis*) were subjected to the above criteria. All isolates grew well at 37°C and pH 3.9 and 9.6, while they varied in growth at 10 and 45°C. However, all failed to grow after 3 h exposure to gastric juice at pH 2.0 but growth variations were obsereved after 3 h exposure at pH 3.0 followed by 4 h exposure to simulated intestinal juice of pH 8.0. *Lactobacillus fermentum* isolates M 1, M 2, M 4 recorded best survival rates. NaCl was tolerated by all isolates whereas elevated concentrations affected growth differently to the point of inhibition at 10%. Bacteriocin like activity was highest by *Lactobacillus paracasei* ssp. *paracasei* M 27 against MRSA and lowest by *Lactobacillus fermentum* M 1 against *Bacillus* cereus and *Salmonella typhimurium*. Testing for antibiotic susceptibility showed 6 out of 14 strains to be resistant to all antibiotics under study. However, *Lactobacillus paracasei* ssp. *paracasei* M 15 was sensitive to all except tetracycline. Other isolates varied, being susceptible to between 1 and 4 antibiotics. These results show the *in vitro* probiotic potential of *Lactobacillus* isolates from camel milk and further *in vivo* investigations are needed.

Key words: Camel milk, probiotics, *Lactobacillus*, functional characteristics.

INTRODUCTION

Lilly and Stillwell (1965) were the first to address meaning "for life", thus conferring beneficial health effects to humans and animals. Recent literature refers to probiotics as viable, non-pathogenic microorganisms that when administered in adequate amounts confer a health benefit on the host (Argyri et al., 2013; Lavilla-Lerma et

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al., 2013). Among the commonly used probiotic bacteria are different species of the genera *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Lactococcus*, *Enterococcus* and very few probiotic yeasts (Morrow et al., 2012; Zheng et al., 2013). Although ample evidence exists supporting functionality of some probiotic strains (Kotzamanidis et al., 2010), this cannot be extrapolated to others without experimentation (Bao et al., 2010; Huang et al., 2013). However, it is well established that intake of probiotic formulations stimulate growth of beneficial bacteria and reduce pathogen activity thus improving the intestinal microbial balance of the host (Chiang and Pan, 2012). Prado et al. (2008) reported certain probiotics to relieve symptoms of inflammatory bowel diseases, colitis, constipation and reduce liver, breast and colon cancers (Zhu et al., 2011; Lee et al., 2011).

Foods fortified with probiotics received expanding market interest as health promoting functional foods (Argyri et al., 2013). To achieve the expected health benefits, such probiotic foods need to contain adequate amount of live bacteria no less than 10^7 CFU/g (Pundir et al., 2013). However, there are some literatures on beneficial immunological effects derived from non viable bacterial probiotic cells (Morrow et al., 2012; Tulini et al., 2013).

An ample amount of literature reported that the most suitable matrices to deliver probiotics are dairy products both fresh and fermented including milk, yoghurt and cheese (Granato et al., 2010; Mahasneh and Abbas, 2010).

Lactic acid bacteria forms the corner stone for probiotics use and no doubt lactobacilli represent the fundamental group (Rivera-Espinoza and Gallardo-Navarro, 2010). Although, there is a drive towards non-dairy and novel probiotics where traditional fermented foods would form an area of search for new probiotic-type functional foods (Sánchez et al., 2012), it is thought that some unusual traditional dairy foods are prospective mining areas for unique probiotics. Among such traditional foods, which did not receive the necessary attention are camel's milk and its fermented products (Mahasneh and Abbas, 2010). In dairy industries, well-adapted commercial starters fail to compete metabolically with wild strains that out-grow others in traditional fermentations (Argyri, et al., 2013). It is recognized now that results obtained from understanding traditional fermented foods would help in securing new probiotics for a wide array of applications (Lavilla-Lermaet al., 2013)

In the last few years, great deal of research on probiotic lactobacilli isolated from novel fermented foods including exotic dairy products was successfully carried out (Bao et al., 2010; Espeche et al., 2012; Monteagudo-Mera et al., 2012). The objective of this study was to isolate and identify selected *Lactobacillus* strains originating from fresh and fermented camel's milk and to study some of their functional properties, antimicrobial abilities against pathogens and survival in certain gut related conditions. This was carried out in an effort to establish their prospective probiotic potential.

MATERIALS AND METHODS

Collection of milk samples and enrichment for indigenous bacteria growth

Ten samples of raw camel's milk were collected directly from camel herds in Jordan. Aliquots of each fresh sample were used and the remainder was allowed to ferment spontaneously at room temperature (25 - 30°C)

The enrichment process of the collected samples was carried out by inoculating 80 ml of MRS broth with 10 ml of the fresh milk samples and incubated anaerobically at 37°C for 5-7 days. All samples were collected into sterile plastic bottles, kept on ice and were transported to the microbiology laboratory within 2 h of collection.

Isolation of *Lactobacillus* strains

Fresh or fermented camel's milk samples were serially diluted in sterile saline and 100 µm were then plated onto de Man Rogosa and Sharpe agar (MRS, Oxoid, UK). Plates were incubated anaerobically using anaerogen bags (AnaeroGen, UK) at 37°C for 2-5 days. Presumptive *Lactobacillus* colonies were randomly picked off the MRS plates and were subcultured onto fresh plates of the same medium to ensure purity. To enhance the chances of isolating *Lactobacillus* strains, MRS medium was supplemented with 0.5 g/L cysteine-HCl (Gomes et al., 1998).

Identification of bacterial strains

All isolates were tested for catalase and oxidase activity, Gram reaction, cell morphology and spore formation (Guessas and Kihal, 2004; Ashmaig et al., 2009). All Gram positive and catalase negative rods were tested for growth in MRS broths at 10, 37 and 45°C (Togo et al., 2002) and for growth at pH 3.9 and 9.6 (Ammor et al., 2005).

The strains were tested for production of acids from carbohydrates and related compounds by using API 50 CH kits and CHL media (BioMérieux, France). The API test strips were prepared according to manufacturer's instructions. Results were scored after incubation for 24 and 48 h at 37°C. These results were joined to the apiweb™ identification software with database (V5.1), which uses the phenotypic data to predict a species identity. Interpretations of the fermentation profiles were facilitated by comparing all results obtained for the tested isolates with information from the computer-aided database.

Maintenance of bacteria

Bacterial cultures were maintained in MRS broth with 20% glycerol and kept stored at -80°C. Working cultures were kept on MRS agar plates or slants stored at 4°C and were routinely sub- cultured every four weeks. For comparative purposes, *Lactobacillus reuteri* DSMZ 20056 purchased from the German Microbiological Collection and known as a probiotic strain was included in some tests.

Preparation of simulated gastric and intestinal juices

Simulated fresh gastric and intestinal juices were prepared daily by

suspending pepsin (P 7000-25G) (Sigma-Aldrich, USA) (0.3% w/v) and pancreatin USP (P-1500) (Sigma-Aldrich, USA) (0.1% w/v), respectively, in sterile NaCl (0.5% w/v) and adjusting the pH to 2.0 and 3.0 for gastric juices using HCl, and 8.0 for intestinal juice with 0.1 mol/L NaOH using pH meter (Eutech 510, Singapore).

Bacterial tolerance to simulated gastric and intestinal juices

Overnight bacterial cultures (30 ml) grown in MRS broth were adjusted to 0.5 McFarland and were centrifuged (2500 × g, for 20 min, at 5°C). The pellets were then washed twice in 50 mM K_2HPO_4 (pH 6.5) and finally were resuspended in 3 ml of the same buffer. One milliliter aliquots of this cell suspension were harvested by centrifugation (12,000 × *g*, for 20 min, at 5°C) and resuspended in 9 ml of gastric solution pH 2 and 3. Total viable counts on MRS plates were recorded, both before and after incubation period of 3 h at 37°C. Then, one milliliter of gastric juices pH 2 and 3 were taken and added separately to 9 ml each of intestinal solution pH 8. Total viable counts on MRS plates were also recorded, after an incubation period of 4 h at 37°C. The results were expressed as colony counts (log_{10} orders CFU/ml).

Determination of total viable counts

Total viable counts of *Lactobacillus* species were determined by spread plate method using MRS agar. Serial tenfold dilutions were prepared in sterile normal saline. Triplicate plates of each suitable dilution were inoculated with 100 µl each and incubated anaerobically (AnaeroGen, UK) at 37°C for 48 h after which numbers of CFU/ml were determined.

Detection of bacteriocin like activity of the bacterial isolates

Preparation of cell-free supernatant of the bacterial isolates

The antibacterial activity of neutralized cell-free supernatants was determined using the agar well diffusion assay. Filter sterilized (0.22 µm syringe filter (Macherey-Nagel, Germany)) cell-free supernatant was obtained from 36 h culture of the selected *Lactobacillus* isolates grown in MRS broth at 37°C under anaerobic conditions. This sterile supernatant was used for the agar well diffusion assay.

Agar well diffusion assay

Bacteriocin-like antibacterial activity was assayed by the agar-well diffusion. One hundred microliters of culture filtrates of selected *Lactobacillus* isolates prepared as above were introduced in triplicates into 8 mm diameter wells of a plate of Müeller-Hinton agar. These plates were previously inoculated with 100 µl of approximately 10^7 CFU/ml of an overnight culture of indicator strains. These strains included (*E. coli* (ATCC 25922), *S. typhimurium* (ATCC 14028), *B. cereus* (Toxigenic strain, TS), and MRSA (clinical isolate). Müeller-Hinton broth cultres of these were adjusted to 0.5 McFarland and were then diluted 1:10 using the same broth. The plates were placed initially at 4°C for 1 h to allow the diffusion of the cell free supernatant and were then incubated aerobically at 37°C for 24 h. Inhibition zones diameter were recorded as positive if the diameter of the zone was 1 mm or larger.

Antibiotic susceptibility testing

The antibiotic susceptibility test was done according to the agar diffusion method published by the National Committee for Clinical Laboratory Standards (NCCLS, 2000). The determination of minimum inhibitory concentration (MIC) to certain antimicrobial agents recommended by Scientific Committee on Animal Nutrition (SCAN, 2002) included ampicillin, ciprofloxacin, erythromycin, gentamycin, kanamycin, streptomycin, tetracycline and trimethoprim. Müeller-Hinton agar (Merck, Darmstadt, Germany) plates were used and incubated under anaerobic conditions. Serial dilutions of antibiotics were prepared using distilled water and were sterilized using 0.22 µm syringe filters (Macherey-Nagel, Germany). One milliliter of each suitable antibiotic concentration was added to 9 ml of molten agar, mixed thoroughly, and poured into sterile petri dishes. The agar plates were allowed to set at room temperature. Bacterial inoculum was prepared by suspending several bacterial colonies from a fresh agar plate in normal saline to a McFarland 0.5 turbidity standard. The 0.5 McFarland suspensions were diluted 1:10 in sterile normal saline to obtain a concentration of 10^7 CFU/ml. A spot of 1 µl of the inocula was placed on the agar surface yielding approximately 10^4 CFU/spot. The inoculated plates were allowed to stand at room temperature for about 30 min. The triplicate plates were transferred into anaerobic jars and were then incubated at 37°C for 24 h. The MIC (Minimum Inhibitory Concentration) was recorded as the lowest concentration of antimicrobial agent that completely inhibited growth.

Statistical analysis

The results are presented as means ± S.D. Statistical differences among bacterial isolates were determined by two way ANOVA except for tolerance to simulated gastric and simulated intestinal juices which were determined by three way ANOVA. Differences were considered significant at $p < 0.05$.

RESULTS

Isolation and identification of *Lactobacillus* potential probiotic strains

A total of 400 isolates from fresh and fermented camel milk samples were cultured using MRS medium. Of these isolates only 34 were Gram positive rods, catalase and oxidase negative and non-spore formers and tentatively presumed to be *Lactobacillus* species. These isolates were further characterized using API 50 CH strips. Results of the API 50 tests confirmed the identity of the 34 *Lactobacillus* isolates (Table 1) which were coded M 1 to M 34. As Table 1 shows the substrate utilization results of API 50 test showed that camel's milk originated *Lactobacillus* isolates were mostly identical in their biochemical and carbohydrate fermentation profiles and they were unable to utilize L-xylose, adonitol and L-rhamnose except M 6, M 7, M 21, M 29, M 31 and M 32. These profiles differed slightly for some isolates compared with the reference strain *Lactobacillus reuteri* (DSMZ 20056) which was purchased from the german microbiological collection (DSMZ) and known for its probiotic properties (Forsberg et al., 2013).

Temperature, NaCl and pH effect on the growth of potential probiotic isolates

The majority of the selected *Lactobacillus* isolates were

Table 1. Summary of API 50 identification results. All isolates were Gram positive rods, catalase and oxidase negative and non-spore formers.

Isolate	API 50 CH Profile																Designated species
	LARA	RIB	LXYL	ADO	GAL	MNE	RHA	MAN	SOR	ARB	ESC	CEL	LAC	MEL	RAF	TUR	
M 1	-	+	-	-	+	v	-	-	-	-	w	-	+	+	+	-	*Lactobacillus fermentum*
M 2	-	+	-	-	+	v	-	-	-	-	w	-	+	+	+	-	*Lactobacillus fermentum*
M 3	-	+	-	-	+	v	-	-	-	-	v	-	+	+	+	-	*Lactobacillus fermentum*
M 4	-	+	-	-	+	v	-	-	-	-	v	-	+	+	+	-	*Lactobacillus fermentum*
M 5	+	+	-	-	-	-	-	-	-	-	+	-	-	v	-	-	*Lactobacillus brevis*
M 6	-	+	-	-	+	+	+	+	v	+	+	+	+	+	+	-	*Lactobacillus rhamnosus*
M 7	+	+	-	-	+	+	v	+	-	+	+	+	+	-	-	+	*Lactobacillus plantarum*
M 8	-	+	-	-	+	+	-	+	-	+	+	+	+	-	-	+	*Lactobacillus plantarum* 1
M 9	+	+	-	-	+	+	-	+	-	+	+	+	+	+	+	+	*Lactobacillus plantarum* 1
M 10	-	+	-	-	+	+	-	+	+	+	+	+	+	+	-	+	*Lactobacillus plantarum* 1
M 11	-	+	-	-	+	+	-	+	+	+	+	+	+	-	-	+	*Lactobacillus plantarum* 1
M 12	+	+	-	-	+	+	-	+	+	+	+	+	+	+	w	+	*Lactobacillus plantarum* 1
M 13	-	+	-	-	+	+	-	+	+	+	+	+	+	v	-	+	*Lactobacillus plantarum*
M 14	-	+	-	-	+	+	-	+	+	+	+	+	+	+	-	+	*Lactobacillus plantarum*
M 15	-	+	-	-	-	+	-	+	+	+	+	+	+	-	-	v	*Lactobacillus paracasei* ssp. *Paracasei* 1
M 16	-	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	*Lactobacillus paracasei* ssp. *Paracasei* 3
M 17	-	+	-	-	+	+	-	+	+	+	+	+	v	-	-	v	*Lactobacillus paracasei* ssp. *Paracasei* 2
M 18	-	+	-	-	-	+	-	+	+	+	+	+	+	-	-	-	*Lactobacillus paracasei* ssp. *paracasei*
M 19	-	+	-	-	+	+	-	+	+	+	+	+	v	-	-	v	*Lactobacillus rhamnosus*
M 20	-	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	*Lactobacillus paracasei* ssp. *paracasei*
M 21	-	+	-	-	+	+	+	+	v	+	+	+	+	-	-	-	*Lactobacillus rhamnosus*
M 22	-	+	-	-	-	+	-	+	+	+	+	+	+	-	-	-	*Lactobacillus paracasei* ssp. *paracasei*
M 23	-	+	-	-	v	+	-	+	+	+	+	+	+	-	-	-	*Lactobacillus paracasei* ssp. *Paracasei* 3
M 24	-	+	-	-	+	+	-	+	+	+	+	+	v	-	-	v	*Lactobacillus paracasei* ssp. *Paracasei* 1
M 25	-	+	-	-	+	+	-	+	+	+	+	+	+	-	-	+	*Lactobacillus paracasei* ssp. *Paracasei* 1
M 26	-	+	-	-	+	+	-	+	+	+	+	+	+	-	-	v	*Lactobacillus paracasei* ssp. *paracasei*
M 27	-	+	-	-	+	+	-	+	+	+	+	+	+	-	-	+	*Lactobacillus paracasei* ssp. *Paracasei* 1
M 28	-	+	-	-	+	+	-	+	+	+	+	+	+	-	-	+	*Lactobacillus paracasei* ssp. *Paracasei* 1
M 29	-	+	-	-	+	+	v	+	v	+	+	+	+	-	-	-	*Lactobacillus rhamnosus*
M 30	-	+	-	-	+	+	-	+	+	+	+	+	+	-	-	+	*Lactobacillus paracasei* ssp. *Paracasei* 1
M 31	-	+	-	-	+	+	+	+	v	+	+	+	+	-	-	-	*Lactobacillus rhamnosus*
M 32	-	+	-	-	+	+	+	+	v	+	+	+	+	-	-	-	*Lactobacillus rhamnosus*
M 33	-	+	-	-	+	+	-	-	-	-	+	+	-	+	+	+	*Lactobacillus brevis*
M 34	-	+	-	-	+	+	-	+	+	+	+	+	v	-	-	v	*Lactobacillus paracasei* ssp. *Paracasei* 2

(+): Positive reaction; (-): Negative reaction; (w): Weak reaction and (v): Variable reaction.

able to grow very well at 10 and 37°C, however, M 1, M 3, M 4, M 12 and M 28 failed to grow at 10°C (Table 2). At elevated temperatures of 45°C, most isolates grow satisfactorily except for M 25, M 26, M 27, M 30 and M 31 which failed to show any degree of growth (Table 2). As for NaCl tolerance, bacterial isolates were able to tolerate 4-6.5% NaCl except M 1 and M 33 which were unable to grow at 6.5% (Table 2). At 8-10% NaCl most isolates were able to show good growth, however, some variations were recorded. M 1, M 2, M 3 and M 33 failed to grow at both 8 and 10% NaCl concentration. As for 10% NaCl, another five isolates were unable to grow, these included M 4, M 5, M 9, M 11, M 12. The pH effect on growth showed the ability of the majority of the isolates to grow well at pH 3.9 and 9.6 except M 33 which was unable to grow at pH 3.6 while it grew well at pH 9.6.

Table 2. Effect of NaCl, temperature and pH on the growth of *Lactobacillus* isolates.

Isolate	NaCl (%)				Temperature (ºC)			pH	
	4%	6.5%	8%	10%	10	37	45	3.9	9.6
M 1	+	-	-	-	-	+	+	+	+
M 2	+	+	-	-	+	+	+	+	+
M 3	+	+	-	-	-	+	+	+	+
M 4	+	+	+	-	-	+	+	+	+
M 5	+	+	V	-	+	+	+	+	+
M 6	+	+	+	+	+	+	+	+	+
M 7	+	+	+	+	+	+	+	+	+
M 8	+	+	+	+	+	+	+	+	+
M 9	+	+	+	-	+	+	+	+	+
M 10	+	+	+	+	+	+	+	+	+
M 11	+	+	+	-	+	+	+	+	+
M 12	+	+	+	-	-	+	+	+	+
M 13	+	+	+	+	+	+	+	+	+
M 14	+	+	+	+	+	+	+	+	+
M 15	+	+	+	+	+	+	+	+	+
M 16	+	+	+	+	+	+	+	+	+
M 17	+	+	+	+	+	+	+	+	+
M 18	+	+	+	+	+	+	+	+	+
M 19	+	+	+	+	+	+	+	+	+
M 20	+	+	+	+	+	+	+	+	+
M 21	+	+	+	+	+	+	+	+	+
M 22	+	+	+	+	+	+	+	+	+
M 23	+	+	+	+	+	+	+	+	+
M 24	+	+	+	+	+	+	+	+	+
M 25	+	+	+	+	+	+	-	+	+
M 26	+	+	+	+	+	+	-	V	+
M 27	+	+	+	+	+	+	-	+	-
M 28	+	+	+	+	-	+	V	+	-
M 29	+	+	+	+	+	+	+	+	+
M 30	+	+	+	+	+	+	-	+	-
M 31	+	+	+	+	+	+	-	+	-
M 32	+	+	+	+	+	+	+	+	+
M 33	+	-	-	-	+	+	+	-	+
M 34	+	+	+	+	+	+	+	+	+

+, Growth; -, no growth; V, variable result. No growth was recorded at pH 2.

On the contrary isolates M 27, M 28, M 30 and M 31 failed to grow at pH 9.6 but grew well at pH 3.6.

Effect of simulated gastric juice and small intestine transit on the viability of the *Lactobacillus* probiotic isolates

The simulated tolerance test results indicated the inability of all isolates to survive 3 h treatment at pH 2 (Table 3). However, at pH 3 and after 3 h exposure, the viable counts of some isolates (M 1, M 2, M 4 and M 5) increased and were highly tolerant where after 7 h at this pH the numbers of these isolates were not greatly affected (Table 3). At the same time, isolates M 9, M 10, M 12, M 14, M 15, M 18, M 20, M 27, M28, M 29 and M31 were tolerant in the sense of being a good probiotic candidate.

These significant results ($p < 0.05$) indicate real probiotic potential of some of these isolates compared to the reference strain *Lactobacillus reuteri* (DSMZ 20056).

Bacteriocin like activity

The 14 *Lactobacillus* isolates were examined for the

Table 3. Effect of simulated gastric juice and intestinal juice on viability of *Lactobacillus* isolates.

Isolate	Viable count (log CFU/ml ± S.D)		
	Gastric juice (pH 3)		Intestinal juice (pH 8)
	0 h	3 h	4 h
M 1	7.65 ± 0.02	8.97 ± 0.03	7.01 ± 0.09
M 2	8.17 ± 0.1	9.8 ± 0.07	7.9 ± 0.15
M 4	7.63 ± 0.12	7.90 ± 0.04	6.91 ± 0.18
M 5	10.63 ± 0.05	11.0 ± 0.03	7.36 ± 0.04
M 6	10.20 ± 0.07	-	-
M 7	9.93 ± 0.08	-	-
M 8	8.89 ± 0.03	8.50 ± 0.03	-
M 9	10.84 ± 0.05	10.61 ± 0.03	6.59 ± 0.05
M 10	9.15 ± 0.07	8.69 ± 0.06	6.98 ± 0.02
M 11	11.67 ± 0.08	-	-
M 12	8.15 ± 0.05	7.67 ± 0.09	6.15 ± 0.02
M 13	8.10 ± 0.05	7.38 ± 0.02	ND
M 14	7.96 ± 0.06	7.69 ± 0.08	7.45 ± 0.04
M 15	10.18 ± 0.03	10.15 ± 0.06	6.47 ± 0.2
M 16	10.97 ± 0.1	ND	-
M 17	8.74 ± 0.06	-	-
M 18	9.08 ± 0.07	8.99 ± 0.02	7.66 ± 0.05
M 19	10.9 ± 0.04	-	-
M 20	9.31 ± 0.03	9.16±0.05	7.25±0.15
M 21	8.32 ± 0.1	-	-
M 22	9.52 ± 0.02	-	-
M 23	12.16 ± 0.05	7.90 ± 0.05	7.72 ± 0.3
M 24	8.33 ± 0.04	-	-
M 26	9.09 ± 0.13	-	-
M 27	11.16 ± 0.07	7.75 ± 0.02	6.83 ± 0.06
M 28	7.98 ± 0.08	7.74 ± 0.16	6.71 ± 0.2
M 29	8.84 ± 0.13	8.23 ± 0.21	-
M 31	9.60 ± 0.1	8.86 ± 0.2	-
M 32	8.85 ± 0.07	7.02 ± 0.04	-
L. reuteri DSMZ 20056	9.61 ± 0.06	9.38 ± 0.03	ND

Results are shown as (mean of log CFU/ml ± S.D, n=3). ND: Not Determined. Differences between all isolates in resistance pattern were significant at ($p < 0.05$). None of the isolates was able to grow at p H 2 .

bacteriocin like inhibitory activity against *B. cereus*, MRSA, *E. coli* ATCC 25922 and *S. typhmurium* ATCC 14028. Table 4 indicates that the supernatant of M 27 (*L. paracasei* ssp. *paracasei*) possessed the highest inhibitory activity (17.7 mm inhibition zone diameter) against MRSA. The lowest activity of the supernatant was observed with M 1 (*L. fermentum*) isolate against *B. cereus* (9.7 mm) and *S. typhimurium* (10.7 mm). Both M 1 and M 2 were active against *E. coli*. On the otherhand, no activity was recorded for M 4 (*L. fermentum*), M 5 (*L. brevis*) and the probiotic control *L. reuteri* DSMZ 20056 against *E. coli*, *S. typhimurium* or *B. cereus*, however they were active against MRSA (Table 4). These results lead to the assumption that these fourteen isolates are capable of producing different levels of bacteriocin like compounds; however, this needs further substantiation.

Antibiotic resistance

Table 5 shows minimum inhibitory concentrations (MICs) of the 14 *Lactobacillus* isolates tested with different antibiotics of different modes of action. Isolates showing MIC values higher than the MIC breakpoint established by the European Safety Authority (EFSA, 2005) were resistant to Ampicillin (cell wall inhibitor) except for strain M 15 (*L. paracasei* ssp. *paracasei* 1) which was susceptible as it is the case with the control *L. reuteri* DSMZ 20056. When protein synthesis inhibitors (erythromycin, gentamycin, streptomycin and tetracycline) were tested,

Table 4. Antibacterial activity of cell free supernatant (inhibition zones diameter) of the selected *Lactobacillus* species. Bacterial species: *Bacillus cereus* (*B. cereus*); Methicillin resistant *Staphylococcus aureus* (MRSA); *Escherichia coli* (*E. coli*) and *Salmonella typhimurium* (*S. typhimurium*).

Isolate	*B. cereus*	MRSA	*E. coli*	*S. typhimurium*
M 1	9.7 ± 0.58	13.7±1.7	11.3 ± 0.58	10.7 ± 0.58
M 2	12.3 ± 0.58	14.7±0.58	10.7 ± 0.58	13.3 ± 1.2
M 4	0.0 ± 0.0	14.7±0.58	0.0 ± 0.0	0.0 ± 0.0
M 5	0.0 ± 0.0	14.3±1.2	0.0 ± 0.0	0.0 ± 0.0
M9	14.0 ± 1.7	14.3±0.58	12.3 ± 1.5	13.7 ±0.58
M10	14.2 ± 2.0	16.8±0.76	12.3 ± 0.58	14.3 ± 1.5
M12	14.0 ± 1.7	15.8±0.76	13.0 ± 1.0	15.0 ± 1.0
M14	14.8 ± 1.4	16.3±0.58	11.3 ± 0.58	14.7 ±0.58
M15	13.3 ± 0.58	14.3±0.58	11.7 ± 0.58	15.7 ± 1.5
M18	13.0 ±0.00	14.0±1.0	11.3 ± 0.58	13.0 ± 1.0
M20	12.3 ± 2.1	14.7±1.5	13.3 ± 1.2	15.0 ± 2.0
M27	14.3 ± 1.5	17.7±0.58	12.3 ± 0.58	14.0 ± 2.0
M29	12.8 ± 1.0	15.0±1.7	12.0 ± 1.0	13.7 ± 1.2
M31	13.3 ± 0.58	15.7±0.58	12.2 ± 0.76	13.8 ± 1.0
L. reuteri DSMZ 20056	0.0 ± 0.0	12.3± 0.58	0.0 ± 0.0	0.0 ± 0.0

Inhibition zone diameter (mm) of indicator strains, mean ± S.D, n=3.

Table 5. Antibiotic susceptibility of the selected *Lactobacillus* species. [a]The breakpoints for *Lactobacillus* sp. by SCAN category. Minimum Inhibitory Concentration (MIC) equal to or higher than the breakpoint is considered as resistant.(R): Resistant; (S): Susceptible; (A): Ampicillin; (C): Ciprofloxacin; (E): Erythromycin; (G): Gentamycin; (K): Kanamycin; (S): Streptomycin; (Te): Tetracycline and (Tr): Trimethoprim.

Isolate	Antibiotic breakpoint[a] (µg/ml)							
	A (2)	C (4)	E (4)	G (1)	K (32)	S (16)	Te (16)	Tr (32)
M 1	R	R	R	R	R	R	S	R
M 2	R	R	R	R	R	R	R	R
M 4	R	S	R	R	R	S	S	S
M 5	R	R	R	R	S	R	R	R
M 9	R	R	R	R	R	R	R	R
M 10	R	R	R	R	R	R	R	R
M 12	R	R	R	R	R	R	R	R
M 14	R	R	R	R	R	R	R	R
M 15	S	S	S	S	S	S	R	S
M 18	R	R	R	R	R	R	R	S
M 20	R	R	R	R	R	R	R	R
M 27	R	R	R	R	R	R	S	R
M 29	R	S	R	S	R	R	S	R
M 31	R	R	R	R	R	R	S	R
L. reuteri DSMZ 20056	S	R	R	R	S	S	S	S

the same profile of resistance was recorded for all 14 isolates. Variable results were observed, for example M 15 (*L. paracasei* ssp. *paracasei* 1) and M 29 (*L. rhamnosus*) were sensitive to erythromycin, gentamycin, and streptomycin. However, strain M 15 was resistant to tetracycline while M 29 (*L. rhamnosus*) and M 31 (*L.

rhamnosus) were tetracycline sensitive. Regarding the DNA interferring antibiotics (ciprofloxacin and trimethoprim), 11 out of 14 of isolates were resistant to ciprofloxacin and trimethoprim and only strain M 4 (*L. fermentum*) and strain M 15 were sensitive to both. Strains M 4, M 15 and M 29 were sensitive to ciprofloxacin. *L. reuteri* DSMZ 20056 control strain showed a rather different profile where it was sensitive to all antibiotics tested except ciprofloxacin, erythromycin and gentamycin. It is clear from these results that it is difficult to judge whether resistance of probiotics to specific antibiotics is desirable or not depending upon what these probiotic formulations are used for.

DISCUSSION

The eternal connection between health, disease and diet always stimulated the quest for novel products of unique functional properties (Meira et al., 2012). As a result and since camel's milk is an exotic food and may form a potential source of probiotic bacteria (Yateem et al., 2008), this study has been carried out. The main objectives were to isolate *Lactobacillus* strains from fresh and fermented camel's milk samples and to evaluate some of the selected isolates for their beneficial probiotic properties. Following laboratory screening of different camel's milk samples, 34 isolates were found to be Gram-positive rods, catalase and oxidase negative and non-spore forming bacteria. These isolates were assigned as members of the genus *Lactobacillus*. With the use of the API 50 CH Kits, 12% of the isolates were identified as *L. fermentum* and these included M 1, M 2, M 3 and M 4 (Table 1). Twenty three percent of the isolates belonged to *L. plantarum* (M 7, M 8, M 9, M 10, M 11, M 12, M 13 and M 14). Forty one percent were identified as *L. paracasei* ssp. *paracasei* (M 15, M 16, M 17, M 18, M 20, M 22, M 23, M 24, M 25, M 26, M 27, M 28, M 30 and M 34). The API fermentation profile varied with the different species and this is not unusual where similar results are reported (Ashmaig et al., 2009; Suriasih et al., 2012; Diaz et al., 2013; Tulini et al., 2013).

Six isolates (18%), which also showed variations in sugars fermentations, were identified as *L. rhamnosus* and these were M 6, M 19, M 21, M 29, M 31 and M 32. The remainder (6%) of isolates (M 5 and M 33) belonged to *L. brevis,* where unexpectedly, these two isolates showed variations in their fermentation profiles. *Lactobacilli* are considered part of the indigenous microflora of the mammalian gastrointestinal tract and of many other niches and fermented foods (Jara et al., 2011; Neville and O'Toole, 2010; Messaoudi et al., 2013) which may explain some limited variations in these profiles.

Temperature, NaCl and pH tolerance

In this study, the 34 selected isolates were able to grow optimally at 37°C (Table 2). However, variations of growth at 10 and 45°C were observed. M 1, M 3 and M 4 were able to grow at 45°C but not at 10°C and they belonged to *L. fermentum*. Pancheniak and Soccol (2005) isolated *L. fermentum* which grow well at 45°C but poorly at 10°C. Isolates M 25, M 26, M 27 and M 30 all grew at 10°C but not at 45°C and they were identified as *L. paracasei* ssp. *paracasei*. Suriasih et al. (2012) observed similar variations with their *Lactobacillus* isolates and this was related, according to Siezen et al. (2010) and Neville and O'Toole (2010), to different isolation environments. Pundir et al. (2013) isolated lactic acid bacteria from fermented foods which were able to grow at 25, 37 and 40°C.

As for NaCl effect, all isolates were able to grow at 4 and 6.5 % concentrations except M 1 and M 33. At elevated concentrations of 8 and 10%, great variations were recorded. For example, M 1, M 2, M 3, M 4, M 5, M 9, M 11, M 12 and M 33 failed to grow at both concentrations of 8 and 10% NaCl. Hoque et al. (2010) isolated *Lactobacillus*—strains from different regional yoghurts, which tolerated 4-8% NaCl. Pundir et al. (2013) isolated lactobacilli from different foods, which were able to tolerate 1-6.5 % only. Experimental results showed that *Lactobacillus* species isolated from camel's milk were able to grow in acidic pH 3.9 except for M 33 and also to survive at pH 9.6 except for M 27, M 28, M 30 and M 31 (Table 2). Ammor et al. (2005) isolated 36 lactobacilli from dried sausage and found only 4 isolates capable of growing at 3.9 but all grew at pH 9.6. Pundir et al. (2013) reported the ability of *Lactobacillus* isolates to grow at 3.5, 3.7 and 4 pH values but did not mention the alkaline side.

Gastric and intestinal juice tolerance

Considering the fact that most microorganisms are destroyed by the gastric acid (pH 1.4-2.0) in the stomach (Chang et al., 2010), probiotic strains need to exhibit tolerance to such condition for survival. None of the isolates in this study were able to survive gastric and intestinal juices after 3 and 4 h at pH 2.0 (Table 3). However, great variations were observed after 3 h exposure to gastric juice at pH 3 and further 4 h exposure to intestinal juice at the same pH (Table 3). In this context, isolates were grouped into isolates of high tolerance (M 1, M 2, M 4 M 5, and M14). Members of this group were able to grow after 3 h exposure to gastric juice at pH 3 and then their population is slightly declined after 4 h exposure to the intestinal juice. Strain M 2 (*L. fermentum*) presented an increase in the growth of 4000% after 3 h exposure to the gastric juice. Botes et al. (2008) observed an increase in number of some lactobacilli isolates after exposure to simulated intestinal juices. The other group included M 6 to M 32 isolates were less resistant but survived at the rate of (99%) for

strain M 15 (*L. paracasei* ssp. *paracasei*) and lowest (65%) for strain M 23 (*L. paracasei* ssp. *paracasei*3). The highest tolerance rate (93%) for the intestinal juice was for strain M 14 (*L. plantarum*) and the lowest (61%) was recorded for strain M 27 (*L. paracasei* ssp. *paracasei* 1). Horáčková et al. (2011) reported failure of *L. rhamnosus* to tolerate simulated stomach conditions. Argyri et al. (2013) isolated several *L. plantarum*, *L. paracasei* and *L. pentosus* strains, which were highly resistant to low pH and comparable to reference strain *L. casei* Shirota. These results, agree with earlier reports about the survival rate of probiotic bacteria through the digestive tract which was variable and strain dependent (Karasu et al., 2010; Meira et al., 2012; Argyri et al., 2013).

Pathogens inhibition

It is usually expected that potential probiotic lactobacilli would be capable of inhibiting the growth of pathogens (Mahasneh and Abbas, 2010; Khay et al., 2011; Kazemipoor et al., 2012; Rushdy and Gomaa, 2013). In this study, the inhibitory potential of the selected isolates against Gram-positive bacteria (*B. cereus*, methicillin resistant *S. aureus* (MRSA)) and Gram-negative bacteria (*E. coli* ATCC 25922 and *S. typhimurium* ATCC 14028) indicates the significant inhibition showed by the strain M 2 (*L.fermentum*) against MRSA and the lowest activity of M 4 (*L. fermentum*) against *E. coli*. These results were different from these of Coeuret et al. (2004) who observed better results of *L. plantarum* against *Salmonella* species and *E. coli*. The same trend of significant activity of *L. plantarum* isolates against Gram-negative pathogen was reported by Yateem et al. (2008). Soleimani et al. (2010) found that probiotic *L. plantarum* ATCC 8014 was very active against bovine mastitis *S. aureus* and *S. aureus* ATCC 25923. Rushdy and Gomaa (2013) reported substantial activity of *L. brevis* isolate against an array of Gram-positive and Gram-negative bacteria. These results are comparable with our isolates M 9, M 10, M 12 and M 14 (all *L. plantarum*) in being active against MRSA.

Testing the supernatant bacteriocin-like substances of the isolates, strain M 27 (*L. paracasei* ssp. *paracasei*) yielded the highest inhibitory activity against MRSA, while strain M 1 was the lowest against *B. cereus*. Khay et al. (2011) reported the bacteriocin-like activity of lactobacilli from Moroccan camel's milk against Gram-positive pathogens only. Conversely, the probiotic bacteria, despite their origin are capable of inhibiting bacteria by several mechanisms (Rodríguez et al., 2012). Hence, further investigations to define the type of the probable bacteriocin present are an idea we share with Kazemipoor et al. (2012). In this context, probiotic bacteria, despite their origin, are capable of inhibiting pathogens by several mechanisms among which are nutrient competition, antimicrobial production, competitive exclusion, immune modulation and modifications pertaining to toxins and their receptors (Rodríguez et al., 2012).

Antibiotic susceptibility

The antibiotic susceptibility of isolates was studied by using eight antibiotics. Results indicate–resistance to ampicillin except for strain M 15 (*L.paracasei ssp. paracasei*1*)*. Ciprofloxacin resistance was also common except for isolates M4 (*L.fermentum*), M 15 (*L.paracasei* ssp. *paracasei*1) and M 29 (*L. rhamnosus*). Resistance to ampicillin and ciprofloxacin is commonly observed in members of genus *Lactobacillus* (Klayraung et al., 2008). Rojo-Bezares et al. (2006) reported resistance of lactobacilli to ciprofloxacin, gentamycin and other aminoglycosides antibiotics. This inherent resistance is probably due to cell wall structure, membrane permeability and potential efflux mechanisms (SCAN, 2002; EFSA, 2005). Variations in resistance to tetracycline were reported for lactobacilli (Temmerman et al., 2003) and most of our isolates were in this trend, however strain M 15 was sensitive to all eight tested antibiotics except tetracycline. Considering the intrinsic resistance of lactobacilli strains to several antibiotics, one would think that lactobacilli isolated in this study from camel milk would lie in the same category of many *Lactobacillus* probiotic strains.

Conclusively, the results of this study showed that camel's milk is an exotic source for probiotic lactobacilli isolation. These isolates were found to possess functional properties *in vitro* comparable to reference strains and form a suitable material for further studies for their technological characteristics, among which is the claimed protective effect of camel's milk to bacteria during gastric transit. Furthermore, food is the common delivery system for probiotic bacteria and can protect such bacteria during passage through the digestive tract. The probiotic potential of lactobacilli is expected to enhance the nutritional value of foods especially if we know that it is increasingly clear that probiotics in general would provide beneficial impacts even without full colonization of the digestive tract (Ohland and MacNaughton, 2010; Rijkers et al., 2011; Seale and Millar, 2013).

Conflict of Interests

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

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Vol. 8(15), pp. 1638-1644, 9 April, 2014
DOI: 10.5897/AJMR2013.6593
Article Number: 78DAE2E43858
ISSN 1996-0808
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African Journal of Microbiology Research

Full Length Research Paper

Preparation and use of oil formulations of *Beauveria bassiana* and *Metarhizium anisopliae* against *Spodoptera litura* larvae

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Received 29 December, 2013; Accepted 10 March, 2014

***In vitro*, the compatibility of eight vegetable oils, used as components in formulation, was studied in conidia of entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, based in three parameters that are been evaluated: germination rate, vegetative growth and conidiogenesis. Almond oil and gingelly oils at 1, 2 and 3% concentrations showed compatibility with *B. bassiana* as well as *M. anisopliae.* Mustard oil and eucalyptus oils at all three concentrations proved toxic to *B. bassiana* and *M. anisopliae* except at 1% concentration. Sunflower oil, olive oil, coconut oil and castor oils displayed compatibility with *M. anisopliae* and toxic to *B. bassiana* except olive oil and castor oil at 1% concentration. Conidiogenesis appear to be more affected than germination for the sample which displayed toxicity. Compatibility classification in to toxic, moderately toxic and compatible enabled assessment of the tested oils for use in formulations. Conidial formulations of *B. bassiana* and *M. anisopliae* with almond oil/olive oil/gingelly oil/castor oil were used for bioassaying against *Spodoptera litura*. All the four formulations displayed higher mortalities of the target pest compared to unformulated conidia.**

Key words: Entomopathogenic fungi, compatibility, vegetable oils, concentrations, bioassay.

INTRODUCTION

The cutworm *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae), is a major destructive polyphagous pest of subtropical and tropical crops, causing serious economical losses (Rao et al., 1993). Concerns about the negative effects of chemical insecticides have led to emphasis on alternative strategies for pest control. The demand for organically grown food warrants methods that utilize non-chemical inputs for pest control to reduce harmful side-effects of pesticides on public health and environment (Hazra et al., 1998). Pest management involving biocontrol agents is assuming prominence and have been considered as an important strategy in insect population reduction. *Metarhizium anisopilae* (Metchnikoff) Sorokin, *Beauveria bassiana* (Balsamo) Vuillemin and *Paecilomyces fumosoroseus* (Wize) Brown & Smith were recognized as important entomopathogens (Wanida and Poonsuk, 2012; Shoaib et al., 2012; Meikle et al., 2005).

In the field, however, the higher temperature, lower humidity and exposure to ultraviolet (UV) radiation could

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Table 1. Isolates of entomopathogenic fungi obtained from USDA/ARSEF collection used in the study of compatibility.

Isolate	Acc. No.[1]	Fungal species	Insect host	Order	Geographical origin
M20	ARSEF1823	*M. anisopliae*	*Nilaparvata lugens*	Homoptera	India
M48	ARSEF 1882	*M. anisopliae*	*Tibraca limbativentres*	Hemoptera	Brazil
B55	ARSEF 654	*B. bassiana*	*Nilaparvata lugens*	Homoptera	China
B51	ARSEF 502	*B. bassiana*	*Ostrinia nubilalis*	Lepidoptera	China

[1]Accession numbers of strains obtained from the ARSEF-USDA.

be detrimental to the conidia. The situation warrants shift to use of oil-formulation of fungi which showed good results in biological control of insect pests under field conditions (Bateman et al., 1993; Feng et al., 1994; Batta, 2003) Formulations of *M. anisopliae* and *B. bassiana* have been used against *Sitophilus oryzae*, *Rhyzopertha dominica* (Batta, 2008); *Tribolium confusm Duval* (Michalaki et al., 2006); *Anopheles gambiae* and *Anopheles stephensi* (Bukhari et.al., 2011) as biocontrol agents.

Studies of cotton seed oil formulations with *Metarhizium flavoviride* against *Schistocerca gregaria* showed increased of infectivity to insects, even at low humidity and high temperatures (Bateman et al., 1993). Furthermore, oil affords protection to fungal conidia from the UV of sunlight (Moore et al., 1993). A number of vegetable oils have been used at different concentrations for preparing conidial formulations of entomopathogenic fungi. Fifty percent oil formulations of *B. bassiana* (Luz and Batagin, 2005); 10% coconut oil formulations (Perry et al., 2005) and 20% oil formulations of *M. anisopliae* against ticks (Hedimbi et al., 2011); 19% coconut oil and 28% soybean oil formulations of *B. bassiana* against almond bark beetles (Batta, 2007) 50% rapeseed and camella oil formulations of *Zoophthora radicans* against *Plutella xylostella* (Batta et al., 2011).

In the present study, compatibility of eight vegetable oils with *B. bassiana* and *M. anisopliae* conidiospores was studied at three concentrations using germination, vegetative growth and conidiogenesis as parameters. Selected formulations were tested against *S. litura* larvae for understanding efficacy of the formulated conidia.

MATERIALS AND METHODS

Fungal cultures

The strains of *M. anisopliae* and *B. bassiana* were obtained from ARSEF-USDA collection (Agricultural Research Service Collection of Entomopathogenic Fungi - United States Department of Agriculture) (Table 1). The monosporic cultures were maintained on Sabouraud's dextrose yeast extract agar (SDAY) stored at 4°C and subculture of the fungal strains was done at two month intervals. The spores from 14-day-old cultures were used for preparing formulations.

Preparation of formulations

Oil-in-water formulation was prepared by mixing the surfactant mixed oil phase with the spore suspension in aqueous phase. *M. anisopliae* strains were cultured on SDAY for 14 days at 25 ± 2°C, spores were harvested using 0.01% Tween-80 and spore suspensions were prepared by centrifuging the conidia in 0.02% Tween-80 and decanting the supernatant in the centrifuge tubes. The suspension was thoroughly mixed using a vortex mixer, after adding sterile distilled water followed by centrifugation and decantation. The procedure of washing the conidia was repeated three times to eliminate Tween-80. The washed conidia suspended in distilled water, formed the conidial stock 200 µl which was mixed with 9.8 ml of distilled water. Required concentration of conidia was prepared using Neubauer haemocytometer. Oil phase of the conidial samples were prepared with sterilized almond oil, olive oil, sunflower oil, gingelly oil, coconut oil, castor oil, mustard oil and eucalyptus oil at three concentrations (1, 2 and 3%). Triton X - 100 was used as nonionic surfactant, Na_2CO_3 (Sodium Carbonate) as stabilizer and Silicon as antifoaming agent. One percent oil formulation consisted of 1% oil, 1% Triton X - 100, 0.5% silicone, 1% Na_2CO3 and 96.5% of the aqueous phase. For 2% and 3% formulations the concentration of oil as well as surfactants was increased to twice and thrice respectively. The mixtures of these two phases were then homogenized using the magnetic stirrer for 60 minutes, to get a stable formulation**.**

Germination assessment

Fifty micro liters of the oil formulation at 3×10^6 conidia/ml was used for inoculating SDAY plates by spread plate method, and four sterile cover slips were randomly placed on each plate. Plates were sealed with parafilm and incubated at 25±1°C. After 24 h post incubation, 1 ml of formaldehyde (0.5%) was transferred onto each plate to arrest germination as per the method of David et al. (2008). Each cover slip was removed and placed on glass slide for making germinated/un-geminated spore count (500 per each cover slip). For each sample three replicates were observed.

Vegetative growth and conidiation

By using the cork borer, a hole with 5mm was made in the middle of SDAY plate and inoculated with 50µl of the formulation at 3×10^6 conidia ml^{-1} and the plates were sealed with parafilm before incubating at 25 ± 1°C. Colony diameter was recorded on 14th day. For assessment of conidiogenesis, the spores were flushed out from the plates using 10ml of 0.02% Tween-80. Spore count was done using Neubauer haemocytometer and three replicates were maintained for each sample. Data was submitted to ANOVA and means were computed by the Tukey test ($p \leq 0.05$).

Compatibility assessment of the different oils was made using the formula Alves et al. (1998)

$$T = \frac{20(VG) + 80(SP)}{100}$$

Where, vegetative growth (VG) and sporulation (SP) were given in

Table 2. Effect of 2% oil formulation on conidia of *B. bassiana* and *M. anisopliae.*

Oil name	Colony diameter [1]		Conidia number/plate [1]	
	Mean(cm)	Reduction percentage	Mean	Reduction percentage
a). Conidia of *B. bassiana*				
Almond oil	4.27 ± 0.25[c(2)]	15.78	$21.8 \times 10^8 \pm 7.23 \times 10^4$[b]	20.04
Olive oil	4.27 ± 0.32[c]	15.78	$12.7 \times 10^8 \pm 7.00 \times 10^3$[c]	53.38
Sunflower oil	4.13 ± 0.27[d]	18.41	$12.8 \times 10^8 \pm 3.46 \times 10^3$[c]	52.98
Gingelly oil	4.43 ± 0.22[b]	12.49	$18.8 \times 10^8 \pm 3.79 \times 10^4$[b]	31.09
Coconut oil	4.03 ± 0.33[d]	20.38	$11.5 \times 10^8 \pm 5.86 \times 10^3$[d]	57.95
Castor oil	4.63 ± 0.23[b]	8.54	$15.5 \times 10^8 \pm 3.06 \times 10^4$[c]	43.33
Mustard oil	4.17 ± 0.47[d]	17.75	$10.2 \times 10^8 \pm 1.15 \times 10^3$[d]	62.45
Eucalyptus oil	4.03 ± 0.27[d]	20.38	$9.70 \times 10^8 \pm 4.73 \times 10^3$[d]	64.49
Control I	4.83 ± 0.43[a]	4.59	$27.7 \times 10^8 \pm 5.20 \times 10^4$[a]	-1.28
Control II	5.07 ± 0.23[a]	0	$27.3 \times 10^8 \pm 10.0 \times 10^4$[a]	0
b). Conidia of *M. anisopliae*				
Almond oil	4.87 ± 0.27[c]	16.57	$7.4 \times 10^8 \pm 1.67 \times 10^3$[d]	26.55
Olive oil	4.8 ± 0.30[c]	17.71	$8.8 \times 10^8 \pm 2.91 \times 10^4$[b]	12.66
Sunflower oil	5.03 ± 0.29[b]	13.71	$6.05 \times 10^8 \pm 2.91 \times 10^4$[e]	39.92
Gingelly oil	5.23 ± 0.42[b]	10.29	$9.7 \times 10^8 \pm 3.45 \times 10^4$[a]	3.72
Coconut oil	4.7 ± 0.22[c]	19.43	$8.02 \times 10^8 \pm 4.91 \times 10^4$[c]	20.35
Castor oil	5.03 ± 0.49[b]	13.71	$9.37 \times 10^8 \pm 2.91 \times 10^4$[a]	6.95
Mustard oil	4.27 ± 0.22[d]	26.86	$5.57 \times 10^8 \pm 4.36 \times 10^3$[e]	44.67
Eucalyptus oil	4.03 ± 0.29[d]	30.86	$6.05 \times 10^8 \pm 1.86 \times 10^3$[e]	39.95
Control I*	5.43 ± 0.57[a]	6.86	$9.5 \times 10^8 \pm 1.86 \times 10^4$[a]	5.71
Control II**	5.83 ± 0.39[a]	0	$10.7 \times 10^8 \pm 2.60 \times 10^4$[a]	0

1 Mean of three replicates; 2 Means followed by the same letter on column are not significantly different by Tukey test ($p \leq 0.05$); *additives without oil; **without oil and additives.

relation to the control (100%). T value of 0 to 30 = very toxic; 31 to 45 = toxic; 46 to 60 = moderately toxic; > 60 = compatible.

Efficacy test of formulations by laboratory bioassay against *S. litura* larvae

Four oil formulations were prepared using gingelly oil, castor oil, almond oil and olive oil with each of the two strains of *B. bassiana* and *M. anisopliae. S. litura* larvae were treated as a batch of 20 kept in perforated plastic boxes by spray application of 2% oil formulation at 10^8 conidia/ml using automiser. Fresh castor leaves were provided as feed every day and containers were cleaned of insect litter daily. They were placed in an environmental chamber set at 25±1°C. The insects were treated for two consecutive days and controls were treated with an equal volume of water with 0.02% Tween 80®. Bioassays were set up with three replicates for each treatment. Mortality data was collected at 24 h intervals. The dead insects were transferred to moist chambers done by Petri dishes autoclaved with a moist filter paper to facilitate mycosis. Before transferring the dead insects into the chambers, their surfaces were immediately sterilized with 1% sodium hypochlorite followed by three rinses with sterile distilled water. The bioassays were repeated twice. The median lethal time (LT_{50}) was calculated from the cumulative mortality data on each day post treatment, using probit analysis (Finney, 1971) and SPSS-11.

RESULTS AND DISCUSSION

There was a variable reduction in spore germination, in vegetative growth and in conidia production in the different formulations of oil to *B. bassiana* and *M. anisopliae*. Compatibility levels differed significantly between the formulations of the two entomopathogens. Formulations of almond oil and Gingelly at 1, 2 and 3% were compatible with *B. bassiana* and to *M. anisopliae* (Table 2). Mustard oil and eucalyptus oil at three concentrations were classified as toxic to *B. bassiana* and *Metarhizium anisopliae*, except in the concentration of 1% for the fungus *M. anisopliae*. On the other hand, Sunflower oil, olive oil, coconut oil and castor oil formulations showed compatibility with *M. anisopliae* and toxicity with *B. bassiana.* Where as mustard oil and eucalyptus oil showed moderately toxic effects on *B. bassiana* at 1% oil concentration and toxic at 2 and 3% concentrations. On the other hand, with *M. anisopliae,* 1% concentration of oils was compatible while at 2 and 3% moderate toxicity was observed. The antifungal activity of Eucalyptus oil was attributed to its active ingredient citronellal. The antifungal activity of citronellal,

Table 3. "T" values and compatibility classification of eight oils on *B. bassiana* and *M. anisopliae*.

Oil name	T Value					
	1% oil concentration		2% oil concentration		3% oil concentration	
	B. bassiana	*M. anisopliae*	*B. bassiana*	*M. anisopliae*	*B. bassiana*	*M. anisopliae*
Almond oil	88.55(C)[2]	80.81(C)	79.96 (C)	73.45(C)	71.25(C)	63.28(C)
Olive oil	68.97(C)	92.72(C)	46.62(MT)	87.34(C)	43.61(T)	77.58(C)
Sunflower oil	56.64(MT)	63.36(C)	47.02(MT)	60.05(C)	40.04(T)	50.79(MT)
Gingelly oil	74.15(C)	97.44(C)	68.91(C)	96.28(C)	62.12(C)	78.41(C)
Coconut oil	52.07(MT)	87.76(C)	42.05(T)	79.65(C)	39.40(T)	72.70(C)
Castor oil	64.95(C)	94.38(C)	56.67(MT)	93.05(C)	50.18(MT)	75.02(C)
Mustard oil	46.35(MT)	61.87(C)	37.55(T)	55.33(MT)	35.60(T)	48.47(MT)
Eucalyptus oil	44.91(T)	65.59(C)	35.51(T)	60.05(MT)	31.15(T)	51.61(M)
Control I[1]	101.64(C)	94.38(C)	101.28(C)	94.29(C)	99.18(C)	92.06(C)
Control II [2]	100.00	100.00	100.00	100.00	100.00	100.00

C = compatible, MT = moderately toxic, T = toxic, [1]Control I = additives without oil, [2] Control II = without oil and additives.

against several species of *Aspergillus*, *Penicillium* and *Eurotium* (Nakahara et al., 2003), and, that mustard oil were reported (Nielsen and Rios, 2000; Dhingra et al., 2004).

Barring few exceptions, all the formulations at the three concentrations and with *B. bassiana* as well as with *M. anisopliae,* germination recorded more reduction rather than the corresponding values for colony diameter. Number of conidia produced showed correspondence to the vegetative growth of the colony measured in terms of colony diameter rather than to percentage of germination. Except that of almond oil the four formulations showed marked reduction in conidiogenesis compared to germination and vegetative growth in *B. bassiana*. With respect to *M. anisopliae* Sunflower oil, eucalyptus oil and mustard oils showed more reduction in conidiogenesis than germination and vegetative growth. It is evident that non-compatible oils show their adverse effect mainly on conidial production. Hence conidiogenesis can be taken into consideration for deciding compatibility of the given oil. Because in case of *B. bassiana* Almond oil is the only oil which is more compatible (Table 3), and it showed less reduction in conidiogenesis compared to germination. Almond, olive, gingelly, coconut and castor oils displayed high compatibility to *M. anisopliae* and also showed less reduction in conidiogenesis, compared to germination except gingelly and castor oils at 3% concentration (Figure 1).

Except mustard oil, the rest of the oils used contain unsaturated fatty acids such as linoleic acid and oleic acids in different proportions, which have anti-fungal properties. Linoleic acid reduced mycelial growth of *Rhizoctonia solani, Pythium ultimum* and led to a significant reduction in growth of *Crinipellis perniciosa* (Walters et al., 2004). Coconut oil contains 90% saturated fatty acids, and of these, lauric acid accounts for 45-48% inhibition of spore germination and radial growth of *Aspergillus niger* (Řiháková et al., 2002). Sunflower oil contains lecithin, tocopherols and carotenoids, but according to Muley et al. (2009) essential oils that contain carotenoids showed anti fungal activity. Drastic reduction in germination and conidiogenesis of *B. bassiana* in the formulation with sunflower oil and coconut oil in the present study may be due to antifungal activity of the components. Qualitative and quantitative composition of fatty acid components of vegetable oils, and surfactants as well as of the insect epicuticle were shown to affect development of *B. bassinana* against *Triatoma infestans* (Luz and Batagni, 2005).

The four oil formulations (Almond, olive, gingelly and castor oils) tested against 3rd instar larvae of *S. litura* displayed high mortality values of 94.5 to 98.6 compared to that of unformulated sample (Table 4) and profuse mycosis and sporulation was observed on the dead cadavers (Figure 2). *Metarhizium* strains M20 and M48 showed maximum mortality values of the larvae treated with gingelly oil formulations. On the other hand *Beauveria* strains B51 and B55 demonstrated maximum mortality in the treatment with Almond oil formulation. However LT_{50} values were least (4.58-4.89 days) both in *M. anisopliae* and *B. bassiana* strains in castor oil formulation, compared to that of unformulated sample (6.72 - 7.01 days). The Relative Virulence Indices calculated based on the three parameters; viz: LT_{50}, percentage mortality and percentage mycosis were maximum with castor oil for *Metarhizium* strains and with Almond oil treatment for *Beauveria* strains.

Oil in water formulations of *Isaria tenuipes* and *Nomuraea rileyi* caused high mortality levels against *Spodoptera* spp. (Vega-Aquino et al., 2010). Results of the present experiment clears that oil in water formulation at low concentration (2% oil in water) increases efficacy against 3rd instar larvae of *S. litura* compared to *B.*

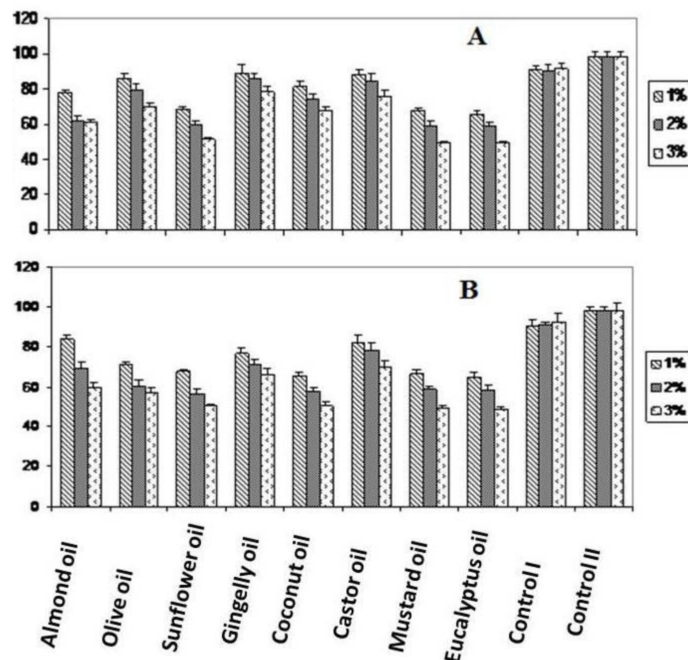

Figure 1. Percentage of conidial germination along with different concentrations (1, 2 and 3%) of vegetable oils after 24 h of incubation, A) *Metarhizium anisopliae*; B) *Beauveria bassiana*.

Table 4. Effect of *Beauveria bassiana* and *Metarhizium anisopliae* oil formulations on 3rd instar larvae of *Spodoptera litura.*

Isolate	Formulation type	LT_{50} (in days)	Mortality%	Mycosis%	RVI*
M. anisopliae ARSEF 1823 (M20)	Almond oil	5.11	$91.5 \pm 0.26^{ab\ (1)}$	94.3 ± 0.67^{ab}	0.12
	Olive oil	5.66	90.3 ± 1.02^{ab}	95.2 ± 0.19^{ab}	-0.07
	Gingelly oil	4.66	98.6 ± 0.52^{b}	96.5 ± 0.19^{b}	0.75
	Castor oil	4.58	97.3 ± 0.38^{b}	96.0 ± 0.68^{b}	0.84
	Unformulated	6.72	85.3 ± 0.21^{a}	75.3 ± 0.41^{a}	-1.31
M. anisopliae ARSEF 1882 (M48)	Almond oil	6.14	90.3 ± 0.58^{ab}	92.5 ± 0.43^{b}	0.06
	Olive oil	6.39	90.2 ± 0.12^{ab}	93.9 ± 0.67^{b}	0.01
	Gingelly oil	5.99	96.7 ± 0.43^{b}	94.3 ± 0.69^{b}	0.65
	Castor oil	4.82	95.2 ± 0.72^{b}	94.1 ± 1.41^{b}	0.92
	Unformulated	7.01	85.1 ± 0.38^{a}	59.1 ± 1.90^{a}	-1.41
B. bassiana ARSEF 654 (B55)	Almond oil	4.69	94.3 ± 0.13^{b}	97.5 ± 0.93^{b}	0.88
	Olive oil	5.63	89.4 ± 0.89^{ab}	70.3 ± 1.90^{c}	-1.61
	Gingelly oil	5.08	93.8 ± 1.01^{b}	95.5 ± 0.49^{b}	0.34
	Castor oil	4.69	92.5 ± 0.64^{ab}	72.5 ± 1.61^{c}	-1.40
	unformulated	6.73	85.4 ± 0.75^{a}	92.3 ± 0.21^{ab}	-1.67
B. bassiana ARSEF 1725 (B51)	Almond oil	4.95	93.1 ± 0.31^{ab}	95.5 ± 0.89^{b}	0.83
	Olive oil	5.72	88.2 ± 0.59^{a}	75.1 ± 1.38^{c}	-1.26
	Gingelly oil	5.18	91.4 ± 1.31^{ab}	94.1 ± 0.69^{b}	0.38
	Castor oil	4.89	90.3 ± 0.59^{ab}	73.9 ± 1.52^{c}	-1.46
	unformulated	6.98	83.3 ± 0.32^{a}	90.4 ± 0.43^{a}	-1.62

([1])Means followed by the same letter on column are not significantly different by Tukey test ($p \leq 0.05$). * Relative Virulence Index.

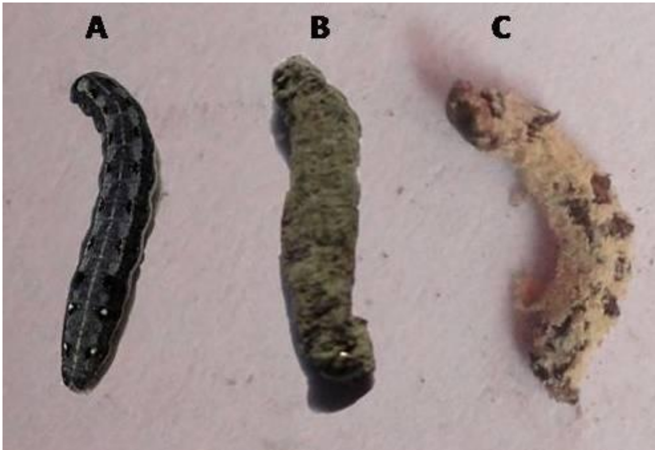

Figure 2. 3rd instar larvae of *S. litura*: A. Control larvae; B. *Metarhizium anisopliae* treated and green sporulated cadaver; C. *Beauveria bassiana* treated and white sporulated cadaver

bassiana strains*.* The insect cuticle contains chitin fibrils within a protein matrix together with lipids, waxes, small quantities of phenols and pigments. Oil formulations promote adherence of spores to the insect cuticle, which facilitate spore germination and subsequent infection process. Successful adhesion depends on the characteristics of mucilage, enzymes, lectins, hydrophobic bonding and electrostatic forces (Boucias et al., 1994). Spore germination is the second step of the infection process followed by formation of an appressorium and many factors have been found to play an important role in conidial germination (Butt, 1990). It is clear from the results that the oil formulations enhance the adhesion of spores to the cuticle and that the low concentration of 2% oil formulations demonstrated mortality rates up to 97% against *S. litura*.

From the present study it is evident that use of formulations at as low as 2% oil generated mortality rates of up to 93% against *S. litura* larvae. formulations with low concentration of oil might prove to be desirable for use at field level.

Conflict of interests

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

ACKNOWLEDGEMENTS

The corresponding author is thankful to Dr. R. A. Humber, ARSEF culture collection Ithaca for providing the strains of *M. anisopliae* and *B. bassiana*.

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Vol. 8(15), pp. 1610-1619, 9 April, 2014
DOI: 10.5897/AJMR2014.6725
Article Number: 1B6BB5C43833
ISSN 1996-0808
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African Journal of Microbiology Research

Full Length Research Paper

Recombinant expression, purification of L-asparaginase-II from thermotolerant *E. Coli* strain and evaluation of its antiproliferative activity

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Received 20 February, 2014; Accepted 17 March, 2014

Repeated use of L-asparaginase II enzyme, in the treatment of acute lymphoblastic leukaemia, is commonly needed because of the enzyme's instability and relatively short half-life which leads to more serious side effects on patients. In the present study, we report on the cloning and expression of L-asparaginase from a thermotolerant strain of *Escherichia coli* (KH027) which was isolated from camel manure and could grow at 45°C. Expression of recombinant asparaginase was conducted by fusion asparaginase gene to pelB leader sequence and 6His residues at the C-terminus under the inducible T7 promoter in DH5α cells. Induction of the cells with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at late log phase of growth resulted in 0.6-fold (2111 UI) higher to that obtained in early log phase induction (1319 UI) and 0.3-fold compared with mid log phase induction (1623 UI). The recombinant asparaginase protein was purified from the culture supernatant through nickel affinity chromatography. The apparent molecular weight of the tetramer enzyme was found to be ~141 kDa. Overall yield (87 mg/L) of the purified recombinant asparaginase was achieved at the shake flask level. The purified protein showed optimum activities at a temperature of 43°C and pH 6. The K_m and K_{cat} parameters were 3.8 mM^{-1} and 2.92 × 10^{3s-1}, respectively. The enzyme retained around 57 and 30% of its initial activity after 30 and 60 min of incubation at 50°C, respectively. Recombinant L-asparaginase was evaluated for its antiproliferative effect in the leukemia cell lines of RS4; 11 and HL60 after 96 and 72 h of incubation. The doses of 100 µg/mL and time-response effect of 96 h caused a reduction value of 50% in cell viability of RS4. However, cell viability of 50% in the leukemic cells HL-60 was noticed with a concentration of 200 µg/mL with an incubation period of 72 h. *In vitro* antiproliferative results in the leukemia cell lines encourage for making *in vivo* investigation to increase the possibility of using this thermostable enzyme in leukaemia therapy.

Key words: L-Asparaginase II, leukemia cell, recombinant asparaginase.

INTRODUCTION

Asparaginases are a cornerstone in the treatment protocols for acute lymphoblastic leukemia (ALL) (Pieters et al., 2011; Muller and Boos, 1998; Keating et al., 1993). L-asparaginase (ASNase) is an enzyme which hydrolyzes amino acid L-asparagine (Asn) to L-aspartic acid and ammonia resulting in the systemic depletion of L-Asn (Horowitz et al., 1968; Goldberg, 1992; Capizzi, 1993; Baran et al., 2002) which in turn induces apoptosis of ALL lymphoblasts (Avramis and Panosyan, 2005; Story et al., 1993; Ueno et al., 1997). Unlike normal cells, cancer cells are unable to synthesize L-Asn due to the absence of L-asparagine synthetase activity (Prager and Bachynsky, 1968; Vieira et al., 2006) where they have L-Asn from extracellular sources (Duval et al., 2002; Keating et al., 1993; Wriston, 1985). Thus, depletion of the circulating pools of l-Asn byl-ASNase leads to the destruction of the tumour cells, since they are unable to complete protein synthesis (Kotzia and Labrou, 2007).

Therapeutic response to those asparaginases of *Erwiniachrysanthemi* and *E. coli* rarely occurs without some evidence of toxicity (Duval et al., 2002; Pavelic et al., 2011) and hypersensitivity reactions (Panosyan et al., 2004; Woo et al., 2000; Larson et al., 1998). Treatment of ALL with L-asparaginase may lead to thrombotic complications and transient protein inhibition in the liver and pancreas (Nowak-Göttl et al., 2003) pancreatitis (Barry et al., 2007; Alvarez and Zimmerman, 2000; Kearney et al., 2009), diabetes, leucopoenia, neurological seizures are associated with the treatment course of ALL with L-asparaginase (Chabner and Loo, 1996; Duval et al., 2002).

Because of many L-asparaginase naturally available, aspraginases are usually not optimally suited for therapeutic purposes, many homologous L-aspraginases have been cloned and characterized to find enzymes with fewer side effects and less toxicity (Kotzia and Labrou, 2005; 2007). Hence, the ideal enzyme for therapeutic use should persist in the circulatory system for long time with reduced antigenic properties. In order to meet these qualities, many attempts have been made to solve this problem by attaching the asparaginase enzyme with chemicals like polyethylene glycol (Burnham, 1994; Veronese and Pasut, 2005; Fernandes and Gregoriadis, 1997), encapsulation to RBCs (Godfrin et al., 2006), deimmunization by combinatorial T-cell epitope removal using neutral drift (Cantor, 2011). Trypsin resistance L-asparaginase with increased stability was achieved by immobilization to natural silksericin protein (Yu-Qing, 2004).

The situation becomes even worse when asparaginase is used repeatedly because of the short half-life and instability that many leads to more serious toxic effects on patients. However, chemical modification usually leads to reduced enzyme activity (Making and Inada, 1975). Therefore, improving the stability of asparaginase without affecting its activity becomes an urgent problem. Some progress has been achieved in protein thermal stability through different trials. These trials give a promising way to enhance thermostability and strengthen the protein’s resistance to proteolysis and improve whole stability of the enzyme (Daniel et al., 1982; Imanaka et al., 1992; Li et al., 2007; Kotzia and Labrou, 2009). In a trail to have a thermostable asparaginase enzyme, our work was directed to have a thermotolerant strain of *E. coli*. We have isolated one from camel's dung in a farm at the desert of A-lkharj governorate in the Kingdom of Saudi Arabia (KSA). In this study we have isolated the (*Ans*B) gene from this strain (KH027) and cloned it in pET20b vector with a C-terminal 6His tag. This is followed by expression, purification, characterization and evaluation of the proliferative activity of the cloned gene.

MATERIALS AND METHODS

Plasmids and bacterial strains

A thermotolerant strain of *E. coli* (KH027) was isolated from camel dung. It was isolated by serial dilution of the dung and its plating into EMB agar medium plates for selecting colonies of the green metallic sheen. Plates were incubated for 24 h at 48°C. Bacterial identification was confirmed by routine biochemical and microscopic tests and API 20 E system (BioMerieux, France). This strain was numbered as KH027 and overnight subcultured in Luria-Bertani (LB) medium and used as a genomic DNA source for cloning step. Cells of the strain of *E. coli* DH5α were used for the cloning of plasmids and as a host in expression studies. The plasmid of pET20b (Novagen) was used as expression vector.

Cloning of L-asparaginase II Gene (AsnB)

The gene coding for the mature region of L-asparaginase II (AnsB) was PCR amplified from the genomic DNA of *E. coli* (KH027) using the two oligonucleotide primers of AsBf (5′-gcggaattcgttacccaatatcacca-3′) and AsnBr (5′-ggcgaagcttgtactgattgaaga-3′). *Eco*RI and *Hind*III restriction sites were incorporated in the primers to facilitate cloning of the asparaginase gene (without its native signal sequence) in the vector pET20b in fusion with by 6His residues at its C-terminus to facilitate the purification step of the recombinant asparaginase protein.

Program of the PCR was conducted as follows: initial denaturation

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at 94°C for 4 min, denaturation at 95°C for 40 s, annealing at 58°C for 30 s, extension at 72°C for 90 s for 35 cycles, and a final extension at 72°C for 10 min. Band of the amplified fragment was 988 bp and extracted from the gel using Qiagen extraction kit and cloned as EcoRI/HindIII digest in pET20b. Ligated product was introduced to competent DH5α cells and the ampicillin resistant transformants were selected and right clones were confirmed by double digestion and insert release. The resultant recombinant plasmid pET20b-Asn-9 His6was sequenced using NCBI BLAST to confirm the asparaginase gene insert**.**

Expression of L-asparaginase II Gene (AsnB)

Transformed cell by clone pET20b-Asn-9 His6 were tested for expression of cloned L-asparaginase II Gene. For this step, 5 mL of LB broth containing 50 µg/mL ampicillin was inoculated by a single colony and grown at 37°C overnight. Fifty milliliters of the LB broth was inoculated with 0.5 mL of the overnight grown culture, and 10 µM of inducer (IPTG) was added after it attained an OD_{600} of 0.6 and grown for a post induction period of 6 h. Cells were harvested by centrifugation at 5000 rpm for 10 min and resuspended in 50 mMTris buffer pH 7 containing lysozyme and the soluble proteins were collected by centrifugation at 11000 rpm for 15 min at 4°C. Extracted protein fractions were mixed with SDS PAGE loading buffer and analyzed on a 12% SDS-PAGE and then stained with Coomassie Brilliant blue R-250. Stained gel was detained to visualize protein bands.

Purification of His-tagged L-asparaginase II Gene (AsnB)

The culture supernatant was collected, by centrifuging the cells at 8000 *g* for 10 min at 4°C, 24 h post-induction, and this was used for purification of recombinant asparaginase by Ni-NTA affinity chromatography. The column was packed with 8 ml of 50% Ni-NTA resin (Qiagen) and equilibrated with equilibration buffer (50 mM potassium phosphate, pH 7.8, 200 mM NaCl, 0.5 mM PMSF, and 20 mM imidazole). The culture supernatant was treated so as to contain 200 mM NaCl, 1 mM PMSF, and 20 mM imidazole and the pH was adjusted to 7.8 by 1M K_2HPO_4. One hundred milliliter of the treated culture supernatant was passed through 0.45 µm filter and loaded on to the Ni-NTA column. The column was washed with 60 ml of equilibration buffer (50 mM phosphate buVer, pH 7.8, 200 mM NaCl, 0.5 mM PMSF, and 20 mM imidazole) and the protein was eluted with elution buffer (50 mM phosphate buffer, pH 8.0, containing 250 mM imidazole and 1 mM PMSF). Fractions of 1 ml each were collected and analyzed by SDS-PAGE and the protein concentration was measured using Bradford dye method (Bradford, 1976) with BSA as a standard. Fractions containing recombinant asparaginase were pooled and dialyzed against 50 mM Tris-HCl, pH 8.0. The protein solution was concentrated by Ultrafiltration using Centricon MWCO 10kDa, Amicon, Germany.

Enzyme assay of L-asparaginase II

Activity assay enzyme was performed according to Imada et al (1973). Samples were mixed with 0.04 M L-asparagine in 0.05 M Tris buffer pH 7.0. 200 µL of assay mixture were incubated at 37°C for 10 min. and the reaction was stopped by adding 50 µL of 1.5 M trichloroacetic acid followed by centrifugation. Nessler's reagent was added to measure the released ammonia after L-asparagine hydrolysis and spectrophotometric measurements were done at 450 nm. Ammonium sulphate calibration curve was used to determine the enzyme activity of recombinant protein and one unit of enzyme activity was defined as the amount of enzyme required to release 1 µM of ammonia per minute under the conditions of the assay at saturating substrate concentration (Wriston, 1985).

Temperature and pH profiles

Activity assay of the purified L-asparaginase enzyme was carried out at different temperatures from 25 to 60°C. For optimum pH, assay of the purified L-asparaginase was conducted with acetate buffer for pH for 4-6, Tris-HCl pH for 7-8 and glycine NaOH for pH 9. Thermostability of the Purified L-Asparaginase: Purified protein of the L-asparaginase was tested for thermal stability at 50°C at different time intervals (10, 20, 30, 40, 50, 60, 70, 80 and 90 min). For this purpose, the pure enzyme was incubated at 50°C for the previous mentioned intervals followed by keeping the enzyme at 4°C. Enzyme activity was calculated at optimum conditions of temperature and pH by L-asparaginase assay. The residual activity was determined by keeping untreated enzyme as control.

Kinetic properties of the purified enzyme

Determining the K_m of the recombinant AsnB Enzyme was performed by incubating the enzyme with different substrate concentrations at different time intervals in 50 mMTris buffer of pH 7.3. Substrate concentrations were taken at the range from 0.2 to 10 mM of L-asparagine. Kinetic parameters Lineweaver-Burk plot (David and David, 1972).

Cell culture and cell preparation

The human leukemia cell lines HL-60 (pre-myeloid leukemia) and RS4;11 (leukemia with lymphoid and myeloid characteristics, Stong et al. (1985) were purchased from the American Type Culture Collection and were maintained in RPMI (GIBCO, USA) supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Human peripheral blood mononuclear cells (PBMC) were purified from heparinized venous blood drawn from healthy donors. PBMC were isolated by centrifugation on Ficoll-Paque (Pharmacia-LKB, Uppsala, Sweden) density gradients (1.077 g/mL) at 1000 rpm for 15 min at room temperature and subsequently re-suspended in RPMI-1640. All cell cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere. The counting and cell viability tests were determined using the test of Try-pan blue exclusion.

Proliferation assay

Cells were seeded in 96-well plates at 1×10^4 cells per well. After 24 h, L-asparaginase was added at concentrations of 15, 25, 50, 100 and 200 µg/mL. At different time points (48, 72, and 96 h) of continuous drug exposure, 10 µl of XTT (XTT II; Roche Molecular Biochemicals, Indianapolis, IN) dye (3 mg/mL) was added in each well. The plates were incubated for 2 h at 37°C and the formazan product was measured at 450 nm by using an iMarkmicroplate reader (Bio-Rad Laboratories). The experiments were performed in triplicate in three independent sets. Values are shown as mean ± SD. Cell survival was calculated by subtracting the background absorbance of media alone and then dividing the absorbance of test wells by the absorbance of the control (untreated) wells.

Miscellaneous

All DNA manipulation protocols, restriction analysis, electro-elution of DNA fragments, ligation, and DNA sequence determination were

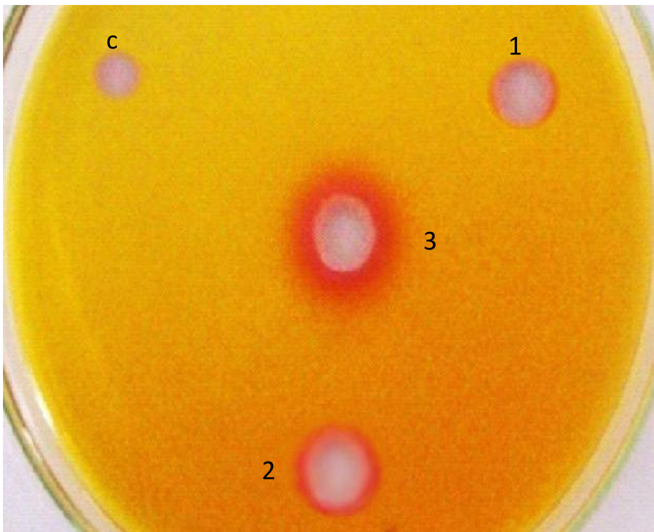

Figure 1. Effect of induction time on the secretion of asparaginase using a rapid plate test for screening L-aspraginase secretion with phenol red as an indicator.cells were grown and induced with 1 mM IPTG at early log phase (1), mid log phase (2), and late log phase (3) of growth and monitored by secretion at zero time (c) as a negative control. Cells were grown for 24 h post-induction and equal amount from each sample (100 µl) was applied in each well.

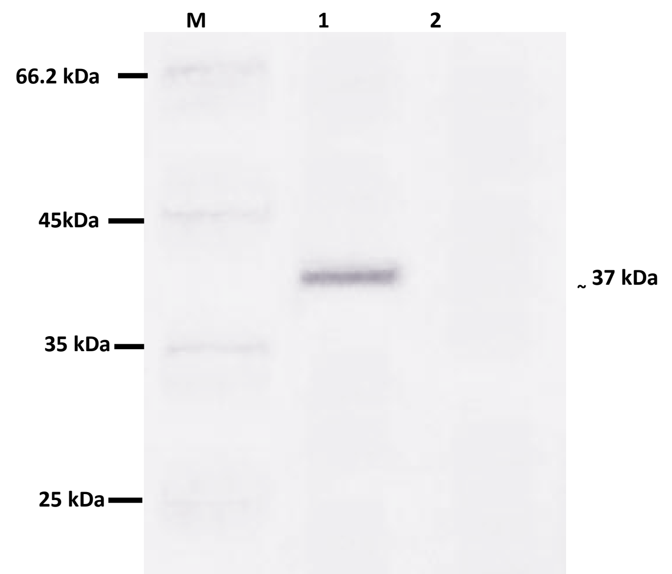

Figure 2. Immunoblotting of the recombinant asparaginase gene. Transformed IPTG-induced cells of *E. coli* strain (KH027) were grown for 24 h on LB medium. Total protein was extracted subjected to 12% SDS-PAGE and Western analysis using anti-His6 antibody. Lane, M: protein marker; Lane, 1: *expressed Asn from clone* pET20b-*Asn*-9; Lane, *3:* proteins of transformed cells with empty pET20b vector as a negative control

performed as described in Sambrook et al. (1989). Polyacrilamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Protein concentrations were determined by the Bradford method (1976). Molecular masses of L-asparaginase were determined by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Garfin (1970). The prestained protein markers (Biolabs, England), broad range 14.4 to 116.2 kDa were used in detection of molecular masses. Immuno-detection of proteins was conducted according to Towbin et al. (1979). Screening of the ability of isolates to produce L-asparaginase was carried out according to Gulati et al. (1997).

RESULTS

Cloning and expression of asparaginase Gene

Genomic DNA of the *E. coli* strain (KH027) was used to amplify the mature part of asparaginase gene by PCR. The amplified fragment (988 bp) was ligated as EcoRI/HindIII digest in pET20b downstream to the T7 promoter and pelB leader sequence. The cloned gene showed 99% similarity with L-asparaginase type II of *E. coli* (K011). IPTG-induced *E. coli* cells, that harboring the recombinant clone pET20b-Asn-9, were grown in LB medium for 24 h post induction. Samples were collected at different stages of growth in order to determine the optimum conditions for asparaginase secretion. For this purpose, cells were induced with 1 mM IPTG at early log phase (OD600:0.6), mid log phase (OD600: 2.0), and late log phase (OD600:4.0) of growth. It was noticed that induction during late log phase resulted in maximum secretion of the recombinant asparaginase (Figure 1). At this stage the total volumetric activity jumped up to (2111 UI) which was 0.69-fold higher to that obtained with early log phase induction (1319 UI) and 0.27-fold compared with mid log phase induction (1623 UI). Also, the specific productivity (total activity/OD_{600}) increased 3-fold with late log phase induction (336UI/OD_{600}) in comparison to early log phase induction (112 UI/OD_{600}). More interestingly the specific activity was also higher. These results show that secretion of the recombinant asparaginase takes place when late log phase induction is done. Therefore, induction during late log phase was chosen in the next experiments of recombinant asparaginase in this study. Also, the molecular mass of the subunit was ~37 kDa consistent with a homodimeric enzyme expression of recombinant asparaginase gene as monitored by immunoblotting using anti-His6 antibody (Figure 2, lane 1).

Purification of recombinant asparaginase

Secreted recombinant asparaginase into the culture supernatant was obtained with IPTG concentrations (5, 10, 25, 50 and 100 µM). Maximum expressed protein in soluble fraction appeared upon induction with 10 µM IPTG (Figure 3, lane 3). The extracellular recombinant

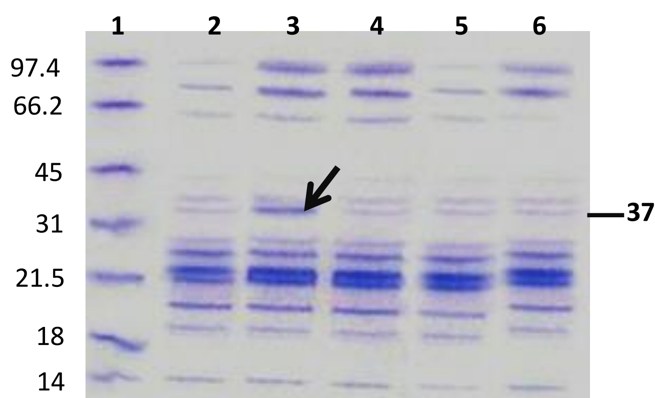

Figure 3. SDS-PAGE analysis of the proteins from soluble fraction after expression. Lane, 1: protein marker; Lanes, 2-6: soluble protein fractions from induced cells with 5, 10, 25, 50 and 100 μM IPTG.

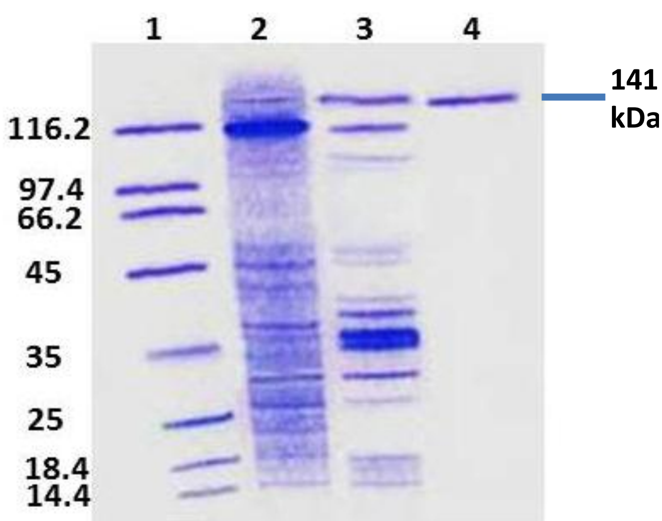

Figure 4. Purification of recombinant asparaginase by SDS-PAGE. Protein samples were analyzed on a 12% polyacrylamide gel. Lane, 1: molecular weight marker; lane, 2: supernatant of the induced transformed culture of *E. coli* BL21 (DE3) bypET20b-Asn-9; lane, 3: supernatant collected after passing through Ni-NTA column; lane, 4: eluted protein from Ni-NTA column after size exclusion chromatography.

protein constituted most of the total extracellular protein. The recombinant protein from the soluble fraction was purified through Ni-NTA column. A single protein band was developed by SDS-PAGE analysis (Figure 4). The apparent molecular weight of the oligomeric protein was ~141 kDa as determined by polyacrylamide gel electrophoresis. An increase of 2.94 fold was achieved upon the purification step where the specific activity of the recombinant asparaginase jumped from 64 to 188

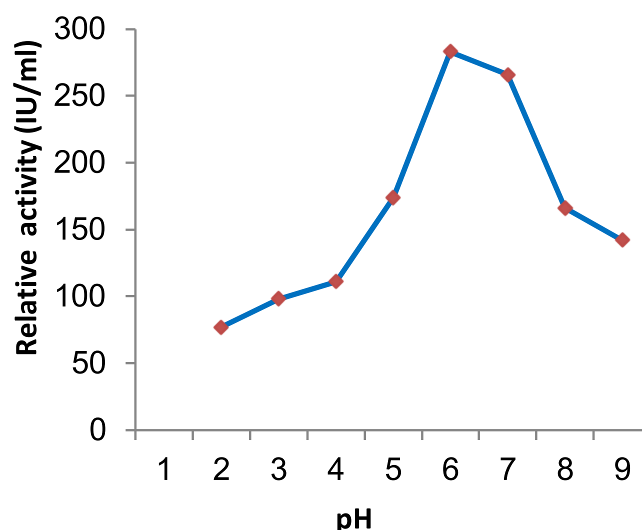

Figure 5. pH profile of the purified recombinant asparaginase. Buffer systems were acetate buffer for pH 4-6, Tris-HCl for pH 7-8 and glycine NaOH for pH 9.

UI/mg. This activity (188UI/mg protein) was close to that of pure asparaginase (200 UI/mg protein), which showed that fairly pure asparaginase preparation has been obtained. Overall yield of 87 mg/L (i.e., 16356 UI/L) of the purified recombinant asparaginase was achieved at the shake flask level.

Characteristics of Purified L-Asparaginase

The purified enzyme was studied for its temperature and pH and profile where it showed activity across a wide pH range with an optimum in the range of 6 to 7 (Figure 5). The activity of the purified enzyme was studied at different temperature degrees (25 to 60°C), exhibited its maximum value at 43°C and decreased beyond 50°C (Figure 6). The thermostability of the purified enzyme was analyzed at 50°C for 90 min and the enzyme retained around 47% of its initial activity even after 30 min of incubation and 24% after 60 min of incubation at 50°C (Figure 7). A sharp decrease was noticed in the thermostability where it retained only 6% of the purified enzyme at 90 min. The affinity of the recombinant L-asparaginase towards the natural substrate L-asparagine was studied and the K_{cat} and K_m values of were investigated as shown in Table 1.

Antiproliferative activity of the purified recombinant L-asparaginase II

Antiproliferative effects of the purified recombinant

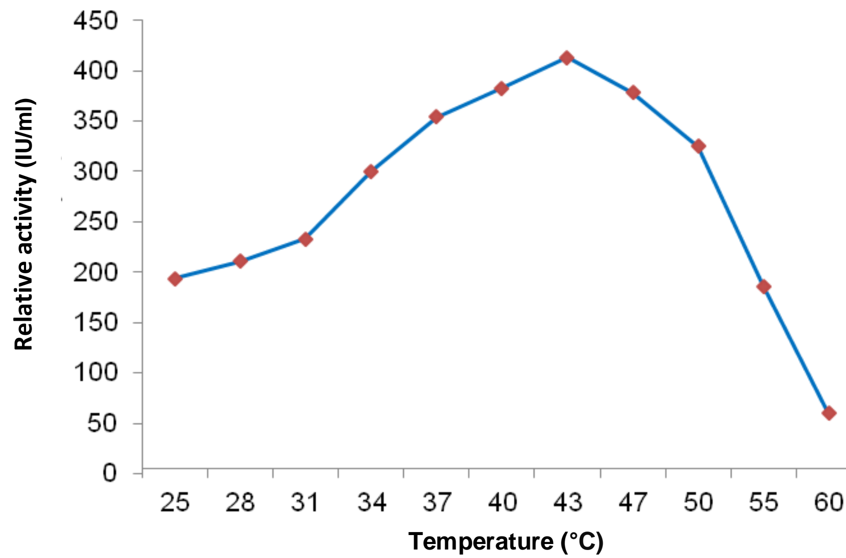

Figure 6. Temperature range for the activity of the recombinant asparaginase. The activity for enzyme was measured at different temperature range (25◦C-60◦C). Asparaginase enzyme showed an optimum temperature at 43°C.

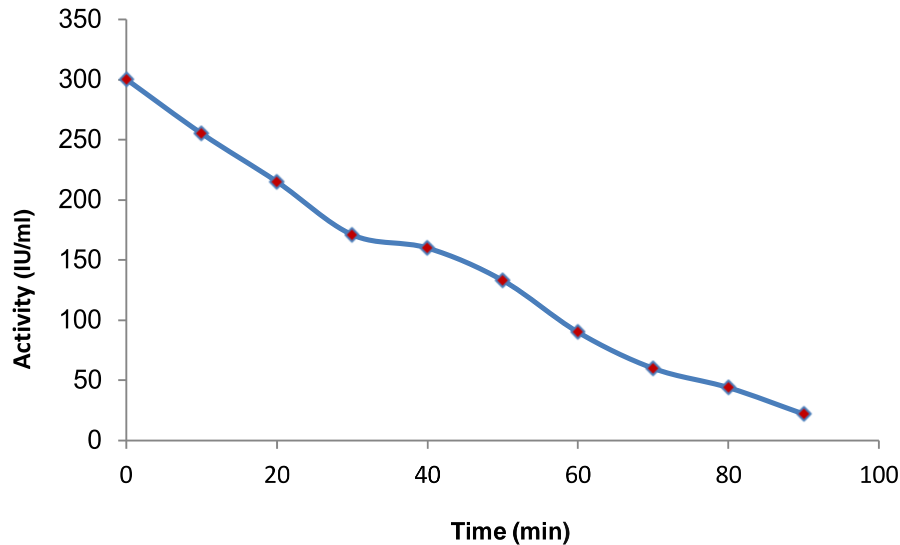

Figure 7. Thermostability of the purified recombinant asparaginase enzyme. At 50°C, a percentage activity retention at various time intervals of (10-90) minutes of incubation.

L-asparaginas from *E. coli* (KH027) was evaluated after 24, 48, 72 and 96 h of incubation against two leukemia cell lines (RS4,11 and HL-60) and peripheral blood mononuclear cells (PBMC). Purified enzyme did not exhibit any observable effect on proliferation of PBMC. In this test, obtained data indicated that the dose of 100 µg/mL and time-response effect of 96 h of incubation are required to cause a reduction value of 50% in cell viability of RS4; 11 (Table 2). Cell viability of 50% in the leukemic cells HL-60 was noticed with a concentration of 200 µg/mL with an incubation period of 72 h (Figure 8). In case of HL-60, it was noticed that the cell viability increased again when the incubation prolonged from 72 to 96 h of incubation (Table 2).

Table 1. Kinetic parameters of the recombinant l-asparaginase II:

E_a (kJ mol^{-1})	K_m (mM^{-1})	K_{cat} ($\times 10^3$ s^{-1})	K_{cat}/K_m ($\times 10^5$ M^{-1} s^{-1})
11.4	3.8 ± 0.1	2.92 ± 0.08	7.68

Table 2. Cell viability of leukemic cells after treatment with purified L-asparaginase from *E. coli* (KH027)

Incubation time (h)	Conentration (µg/ml)	Cell viability (%) RS4-11	Cell viability (%) HL-60
	15	96	83
	25	92	90
48	50	88	88
	100	82	75
	200	76	64
	15	80	77
	25	78	73
72	50	70	68
	100	66	54
	200	59	**50**
	15	73	86
	25	71	83
96	50	68	71
	100	**50**	82
	200	43	63

DISCUSSION

One of the main goals of protein engineering is to produce thermostable enzymes. However, the thermal stability of a protein is not readily predictable from its 3D structure. Directed evolution is the best way to alter this enzyme property (Giver et al., 1998; Oh et al., 2002; Hao and Berry, 2004; Bommarius et al., 2006).Some progresses made in the studies of protein thermostability have shown some promising ways through a single amino acid substitution (Imanaka et al., 1986, 1992).In this work, asparaginase gene of a thermotolerant *E. coli* strain (KH027) was expressed as a His-tagged protein.On sequencing and BLAST analysis, thecloned gene showed 98% similarity with L-asparaginase type II of *E. coli* KO11. Theaddition of histidine tag to the C-terminal region of the gene permits the purification of the recombinant protein through nickel affinity chromatography.

There are very few reports on the efficient release of recombinant proteins into the culture medium of *E. coli*. Although extracellular secretion of recombinant proteins has been achieved by co-expression of permeabilizing proteins, it leads to non-specific secretion and also requires controlled low level expression of the co-expressing gene to avoid problems of cell lysis and lethality (Makrides, 1996; Jonasson et al., 2002). There is no space limitation for the accumulation of the protein, moreover the cells need not be lysed or processed for extracting the desired protein. The extracellular compartment is protease deficient which is important for proteins susceptible to proteolysis, the protein exported to the medium is mostly soluble, biologically active, has an authentic N-terminus and also results in minimization of the potentially harmful action of recombinant proteins against the host cells. In this work we have shown that the use of pelBleader sequence, along with induction strategy significantly improves extracellular secretion of asparaginase to yield reasonably high amounts of protein in the culture medium. Induction at late log phase resulted not only in higher volumetric activity but more importantly promoted secretion specificity, where the recombinant protein constituted a major fraction of the total protein present in the extracellular medium.

Upon SDS PAGE and Coomassie staining ̴37 kDa protein band was visible in induced fractions. It was consistent with a homodimeric enzyme expression of recombinant asparaginase gene. The recombinant protein from the soluble fraction was purified through Ni-NTA column and dialysed fractions. After PAGE analysis, the apparent molecular weight of the oligomeric protein was ̴141 kDa. Maintaining a high specific growth rate, while running a high cell-density culture, is required to enhance the production of recombinant protein. This helps to overcome the metabolic stress associated with recombinant protein expression (Sanden et al., 2003). The specific growth rate has a direct effect on the export of periplasmic proteins into the medium (Shokri et al., 2002). The overall yield of the purified recombinant asparaginase was 87 mg/L (that is, 6.5-fold) compared to the previously reported yield of 10-15 mg/L of purified protein when recombinant asparaginase was expressed in the periplasm (Harms et al., 1991). It should be noted that this level of expression for the recombinant asparaginase was achieved under shaking condition. This means that the extracellular environment is the preferred location for the accumulation of recombinant proteins.

Temperature and pH profiles of the purified enzyme were studied. Results correlate with the earlier reports of

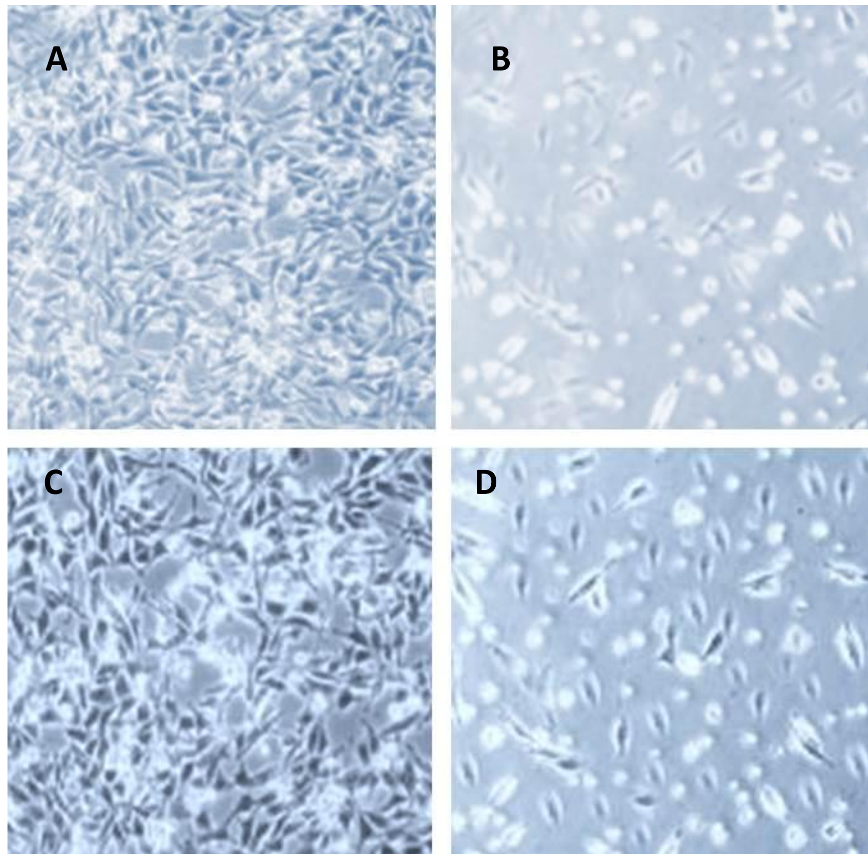

Figure 8. Antiproliferative effect of the recombinant asparaginase enzyme.A and C are cells (1 × 10^4 cell/well) of non-treated leukemic RS4;11 (b) and leukemic HL-60, respectively. B and D are treated cells of RS4;11 HL-60 with of L-asparaginase (5 IU/ml) for 48 h, respectively. Images of were taken after a 48 h-incubation.

L- asparaginase II from *E. coli* and other organisms (Joseph, 1976; Stecher et al., 1999). Based on the control, the enzyme displayed 50 and 23% remaining activity of its initial one even after 60 and 90 min of incubation at 50°C. At 55°C the thermal stability of wild type enzymes of *E. carotovora* and *E. chrysanthemi* displayed 19.9 and 37.2% remaining activity, respectively (Kotzia and Labrou, 2009). Theobtained K_m value is higher than the reported K_m for L-asparagine in the casesof *E. coli* and *Erwinia* enzymes (Kotzia and Labrou, 2009; Christian et al., 2000).

The anti-proliferative effect of the purified enzyme of L-asparaginase produced *E. coli* (KH027) was evaluated. There was no effect on proliferation of PBMC which reflects this activity of L-asparaginase to cancer cells over normal lymphocyte. Reduction in cell viability by 50% in case of HL-60 was achieved after 72 h and after 96 h in case of line RS4;11. Other studies have demonstrated increased asparagine synthetase (AS) expression in cells treated with L-asparaginase which allows these leukemia cells to become resistant to the treatment. Other adaptive processes may provide a substrate to asparagine synthetase (for example, aspartate or glutamine) which comes from intracellular and extracellular sources (Aslanian et al., 2001; Aslanian and. Kilberg, 2001).

Conclusion

In this study, we have isolated L-asparaginase II gene from a thermotolerant *E. coli* strain, cloned in pET20b vector with 6His residues at the C-terminus downstream to the T7 promoter and *pel*B leader sequence, and biochemically characterized. L-asparaginase produced by *E. coli* (KH027) displayed good results on two Leukaemia cell lines. These results prompted for further investigations and can nominate this type of enzymes as candidates in therapy where the pharmaceutical proteins should maintain appropriate serum levels for imparting its complete potency. Apart from the clinical use, this enzyme can be used in fried starchy food preparations to

reduce the acrylamide content, a potent carcinogen formed during the baking process by the reaction of asparagine and sugar at high temperature.

Conflict of interests

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

ACKNOWLEDGEMENT

The authors like to acknowledge the deanship of scientific research at the University of Salman bin Abdul-Aziz, KSA for their kind financial support of this work (Grant No. 29/S/1433).

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Vol. 8(15), pp. 1620-1626, 9 April, 2014
DOI: 10.5897/AJMR2013.6438
Article Number: F25046443842
ISSN 1996-0808
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African Journal of Microbiology Research

Full Length Research paper

Environmental impact on postoperative wound infections in a privately owned hospital in Ghana

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Received 20 October, 2013; Accepted 27 December, 2013

Edwin Cade Hospital of Anglogold, Ashanti, had been experiencing high levels of surgical site infections, but the causes of these were unknown. This study aimed to investigate environmental contamination and postoperative wound infections in the hospital. Wound samples were collected from infected surgical sites and also environments of operating theatre and the surgical wards and cultured for bacteria. Growths on culture media were identified. Antimicrobial susceptibility of the isolates was tested. Culture results indicated bacterial infections of 86% of the surgical site wounds sampled. Wounds in female surgical ward had 92%, male surgical ward had 84.2% and the maternity ward had 81.4% wounds infected with various bacterial types. The most occurring isolate was *Staphylococcus aureus* (54.3%) followed by *Escherichia coli* (16.3%), Coagulase-negative *Staphylococcus* (15.5%), *Proteus mirabilis* (7.8%) and *Pseudomonas aeruginosa* (6.2%). Similar isolates were obtained from the environmental samples with *Bacillus sp.* (43.9%) dominating followed by *S. aureus* (24.9%). There was strong correlation between wounds isolates and environmental isolates (OR=.678, P>0.05). Post-operative wound infection in the hospital was high recording 86% probably due to environmental contaminants. Stronger infection control measures are advocated for the hospital.

Key words: Surgical site, postoperative wound infection, surgical wound, environmental contamination.

INTRODUCTION

There are many diseases that affect humans, which require surgical interventions to correct, but surgical site infections are also common creating serious post-operative complications (de Lissovoy et al., 2009). In the developing world, surgical site infections affect up to two-thirds of patients who undergo operations (Bernstein, 2013) and account for about 20% of all hospital-associated infections (Nasser Abdulsalam et al., 2013). These surgical site infections are common and easily noticeable at Ghana, but data describing the situation in Ghana is scarce. This study therefore aimed at determining bacterial types infection post-operative wounds at Edwin Cade Memorial Hospital, AngloGold Ashanti, Obuasi. It was also to determine whether environmental isolates were responsible for the surgical site infections.

MATERIALS AND METHODS

The study site

The study site was Edwin Cade Memorial Hospital, Obuasi where the study was conducted between July to September, 2011. The study was performed after permission was obtained from the hospital authorities and after ethical clearance was obtained from the Committee on Human Research and Publication of Kwame Nkrumah University of Science and Technology, Kumasi. This study was performed after the informed consent of the patients was obtained. Patients who refused participation were excluded. This is

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Table 1. Characteristics of the patients and the prevalence of surgical site infection.

Sex	Male: n=57	Female: n=93	Total: N=150
Mean age	42.5 ± 14.5	35.4 ± 10.4	35.4 ± 10.4
Number (%)of wounds contaminated	52 (91.2)	77 (88.2)	129 (86)

a prospective on patients of all sexes aged 14 years and above and who had surgery at the Edwin Cade Hospital.

Post-operative wound sampling

After surgery, the dressings were removed and the wounds inspected after 72 h for signs of the presence of pus and malodors. When pus and malodors were present, the wound was considered infected. Infected wounds were sampled with a cotton-tipped swab to collect pus from the infected wound. The swab was placed in Stuarts transport medium and then transported to the bacteriology laboratory for processing. In the laboratory, the specimens were inoculated onto Blood agar and MacConkey agar and incubated aerobically at 37°C overnight. Blood agar and or MacConkey plates which grew less than five (5) colonies for a wound were considered to have mere contamination but not an infection. Dry wound without pus were not sampled and therefore were not included in the study.

Sampling surgical theatre environment

Fomites

Two sample were taken from the fomites. One set of samples was taken before the theatre was cleaned and disinfected and another set was taken just hour after the theatre was cleaned and disinfected. Samples were collected using sterile swabs moistened with sterile saline and used to collect samples from door handles, bedstead, water-taps, and trolleys in the surgical theatre. Each of the samples was placed into a tube containing peptone water. The tubes were labeled appropriately and then taken to the laboratory for analysis.

In the laboratory, the specimens were incubated aerobically at 37°C for 3 h to resuscitate stressed bacteria. After resuscitation, the specimens were sub-cultured onto Blood agar and MacConkey agar and then incubated aerobically at 37°C for overnight and inspected for growth. If no growth occurred, the agar plates were re-incubated for another 24 h. If a culture failed to grow the agar plates were discarded.

Air sampling

Petri dishes containing plate count agar (PCA) were left open for 1 h in the surgical theatre, maternity ward and also the male and female surgical wards. There were five Petri dishes in each of the locations mentioned. The Petri dishes were then taken directly to the laboratory and incubated aerobically at 37°C for 24-48 h. After incubation, growths which occurred on the five plates in each room were counted and the mean count was taken as the count for that site and the organisms were idenfied.

Bacterial identification

After incubation, growths that occurred on the MacConkey and Blood agar plates were identified using, lactose fermentation, formation of mucoid colony forms and other colony forms. Bacterial colonies of similar characteristics were picked and subcultured onto nutrient agar to obtain pure cultures. Bacterial identification was done using the pure cultures on the nutrient agar using conventional methods involving Gram staining and appropriate biochemical tests (catalase, coagulase, oxidase, indole, citrate utilization, urease production, motility test sugar fermentation tests on Triple sugar iron agar tests) and following previously described standard protocols (Cheesebrough, 2000; Gilchrist, 1993).

Antimicrobial susceptibility testing of the isolates

Antimicrobial susceptibility of the isolates was determined by the modified disk diffusion Kirby-Bauer method (CLSI, 2010) on Muller-Hinton agar using the following antibiotic discs: ampicillin (10 µg), co-trimoxazole (25 µg), gentamicin (10 µg), cefuroxime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), Chloramphenicol, penicillin (10 µg), flucloxacillin (5 µg), erythromycin (5 µg), and tetracycline (10 µg). The test was performed by preparing 0.5 McFarland suspension of the test. The suspension was seeded onto Muller-Hinton agar and the antibiotic discs were applied. The set-up was incubated at 37°C overnight. The zone of inhibition around each disc was then measured and compared to a standard chart to determine whether it was susceptible or resistant.

Statistical analysis

The Pearson's χ^2 test, Fisher's exact test, or linear-by-linear association were used to compare categorical variables, as needed. To determine independent risk factors for mortality, a multiple logistic regression model was used to determine the effects of confounding variables. The results of logistic regression analyses were reported as adjusted ORs with 95% CIs. All *P*- values were 2-tailed, and $P < .05$ was considered to indicate statistical significance.

RESULTS

General characteristics of the study population

There were 150 patients (57 males and 93 females) who had surgical procedures to correct various medical conditions at the Edwin Cade Memorial Hospital, AngloGold Ashanti, Obuasi within the period under study. The patients had ages ranging from 16 to 76 years with a mean age 38.1±12.4 years. The majority (91.3%) of the patients were young adults with modal age group of 12-25 years had their wounds contaminated. The mean age males had 42.5 ±14.5 years, which was statistically significant (p=0.001) as compared with female age of 35.4±10.4 years. Of the 150 wounds sampled, 129 grew bacteria, indicating postoperative wound contamination prevalence of 86% (Table 1).

Table 2. Bacteria types isolated from surgical wounds in the ward of admission.

Parameter	Female Surgical	Male Surgical	Maternity	Total
Number of wounds sampled	**50**	**57**	**43**	**150**
Wound isolates	n=46 (%)	n=48 (%)	n=35 (%)	n=129 (%)
Staphylococcus aureus	24 (34.3)	28 (40.0)	18 (25.7)	70 (54.2)
Escherichia coli	9 (42.8.0)	8 (38.1)	4 (19.1)	21 (16.2)
CNS	7 (35.0)	6 (30.0)	7 (35.0)	20 (15.5)
Pseudomonas aeruginosa	4 (50.0%)	2 (25.0)	2 (25.0	8 (6.2)
Proteus mirabilis	2 (20.0)	4 (40.0)	4 (40.0)	10 (7.8)
Total	46 (92.0)	48 (84.2)	35 (81.4)	129 (100)

n (%)= number (frequency) of times a bacterial type was isolated; CNS=coagulase negative staphylococci.

Table 3. Surgical procedure type and prevalence of surgical site infection.

Type of surgical procedure	Number of surgeries performed	Number (%) contaminated
Caesarian section	43	35 (81.4)
Myomectomy	20	19 (95.0)
Total hysterectomy	19	19 (100.0)
Inguinal Hernia Repair	17	15 (88.2)
Hydrocelectomy	14	13 (92.9)
Limb Amputation	13	10 (76.9)
Appendectomy	8	7 (87.5)
Cholecystectomy	7	6 (85.7)
Thyroidectomy	6	2 (33.3)
Colectomy	3	3 (100.0)
Total	150	129 (86)

Surgical wound isolates in relation to ward of admission

Different types of bacteria were isolated from the patients and from all the wards. The most common occurring bacterium was *S. aureus* (54.3%), followed by *E. coli* (16.3%) and Coagulase-negative staphylococcus (15.5%). Other organisms isolated were *P. mirabilis* and *P. aeruginosa* with their respective occurrences of 7.8 and 6.2%. These isolates occurred at high prevalence levels across all the surgical wards from which samples were collected as presented in Table 2.

Surgical procedure in relation to postoperative wound infection

There were 43 caesarian births, being the most frequent surgeries performed in the hospital, out of which number 43/35 (81.3%) had infections. Out of the 20 myomectomy cases, 19 (95%) were infected but all the 19 patients who had total hysterectomy had their wounds contaminated with various bacterial types. Other surgical procedures that had infections have been presented in descending order of frequency as shown in Table 3.

Environmental contamination in relation to surgical wound infection

Various microorganisms were isolated from the fomites in the wards with *Bacillus sp.* having the most common prevalence levels of 50, 38.1, 42.1 and 48.5% in the male surgical ward, female surgical ward, maternity ward and theatre respectively (Table 4). *S. aureus* was the second most common isolate in the wards, registering 27.8, 14.3, 26.3 and 38.8% respectively in the male surgical ward, female surgical ward, maternity ward and theatre. Tables 5 and 6 indicate levels of bacterial contamination at the various parts of the surgical ward and the theatre. The bacteria types were more frequently isolated on the ward than from the theatre. Comparing level of contamination of fomites at the two sites (Surgical wards and the Operating theatre) Bacillus sp. and *S. aureus* and *E. coli* were again the most common isolates. Other isolates and their frequency of isolation are indicated in Tables 5 and 6.

Prediction that a bacterial type isolated from a wound is an environmental contaminant

Prediction that an isolate was obtained from the wound

Table 4. Distribution of bacteria isolated in wards using exposed plates for 1 h.

Isolates	Male ward	Female ward	Maternity ward	Operating theatre	Total
	N (%)	N (%)	N (%)	N (%)	N (%)
Bacillus sp	9 (50)	8 (38.1)	8 (42.1)	5 (48.5)	30 (43.9)
S. aureus	5 (27.8)	3 (14.3)	5 (26.3)	4 (38.8)	17 (24.9)
E. coli	2 (11.1)	5 (23.8)	2 (10.5)	0	9 (13.2)
Klebsiella pneumoniae	2 (11.1)	5 (23.8)	2 (10.5)	0	9 (13.2)
P. aeruginosa	0	0	2 (10.5)	0	2 (2.6)
CNS	0	0	0	1 (9.5)	1 (1.5)
P. mirabilis	0	0	0	1 (9.5)	0.3 (0.4)
Total	18 (100)	21(100)	19 (100)	11 (9.5)	69 (100)

N, mean viable count (%); CNS-Coagulase Negative S*taphylococcus, E. coli*, *S. aureus, P. aeruginosa.*

Table 5. Bacteria isolated from fomites in the surgical wards using swabs.

Organisms	Main door handle N/ (%)	Bathroom door handle N/ (%)	Lavatory door handle N/ (%)	Ward Tap N/ (%)	Bath Tap N/ (%)	Bed N/ (%)
S. aureus	7 (38.9)	7 (38.9)	9 (42.9)	3 (16.7)	2 (12.5)	9 (15)
Bacillus sp.	7 (38.9)	7 (38.9)	9 (42.9)	7 (38.9)	5 (31.3)	34 (56.7)
K. pneumoniae	3 (16.7)	1 (5.6)	1 (4.8)	4 (22.2)	5 (31.3)	6 (10)
E. coli	1 (5.6)	3 (16.7)	2 (9.5)	4 (22.2)	4 (25)	11 (18.3)

was environment gave Odds Ratio (OR) and P values suggesting strong correlation. Results obtained in the χ^2 test predictions for predicting *S. aureus* isolated from the environment and wounds from male patients gave OR= 0.942, and p=.023 for *S. aureus* indicating strong correlation. There was a similarly strong correlation between male patient *E. coli* isolates and that of male ward environment (OR= 0.896, P= .012). Isolates obtained from female wounds were compared with theatre environment isolates *S. aureus* (OR= 0.746, P= .012); wound *P. mirabilis* (OR= 0 .996, P= .023) had a correlation suggesting that postoperative wound infection might be as a result of contamination in the theatre. Maternity ward environmental isolates were compared with postoperative wound isolates from the maternity patients with respect to *P. aeruginosa* and *S. aureus* and there were strong correlations (OR .996, P= .002); (OR .811, P= .042) respectively. These results and other comparisons are presented in Table 7.

Antimicrobial susceptibility of isolates

Antimicrobial susceptibility patterns of the isolates obtained in the study are varied and are presented in Table 8.

K. pneumoniae

K. pneumoniae isolated from the environmental samples had resistant strains to almost all the antibiotics tested while all the *K. pneumoniae* isolates (100%) were resistant to ampicillin and tetracycline; proportion to resistant to cotrimoxazole and cefotaxime were 90.5 and 75.0% respectively. The resistance levels to antimicrobials chloramphenicol and ceftriaxone was 60%, and were 40% to gentamycin and 30% to cefuroxime.

E. coli

E. coli strains isolated from the post-operative wounds had resistant strains to eight antibiotics tested. While all (100%) of the *E. coli* isolates were resistant to tetracycline and ampicillin, resistance level to cefuroxime was 81% and cotrimoxazole was 66.7%. The resistance levels to other antimicrobials were much lower as shown in Table 8.

P. mirabilis

No *P. mirabilis* isolate was susceptible to tetracycline and cotrimoxazole, but their resistant proportion to ampicillin and cefotaxime were both 90%. Proportions resistant to other antimicrobials were 80% to cefotaxime, 60% to cefuroxime, 50% to gentamycin and chloramphenicol, 50%.

Coagulase negative staphylococci

Ampicillin and flucoxacillin had all the coagulase negative *staphylococcus* isolates to them (100%) while resistance

Table 6. Bacteria isolated from fomites in the operating theatre.

Organism	Main door handle n/ (%)	Theatre tap n/ (%)	Theatre bed n/ (%)	Trawley n/ (%)	Reagent Bench n/ (%)	Total N/ (%)
S. aureus	4 (66.7)	1 (16.7)	1 (33.3)	1 (16.7)	3 (50)	6 (100)
CNS	0	0	0	1 (16.7)	0	0
Bacillus sp	2 (33.3)	5 (83.3)	1 (33.3)	4 (66.7)	3 (50)	0
Proteus mirabilis	0	0	1 (33.3)	0	0	0

CNS, Coagulase Negative *Staphylococci.*

Table 7. Prediction that a wound isolate is an environmental contaminant.

Parameters compared	Isolates			
	S. aureus	***E. coli***	***Proteus***	***Pseudomonas***
Location versus the environment	OR (P)	OR (P)	OR (P)	OR (P)
Male wound vrs theatre environment	0.942 (0.023)	--	.996 (.003)	--
Male wound vrs male ward environment	0.746 (0.012)	.896 (.012)		--
Female wound vrs theatre environment	968 (0.023)	--	.426 (.120)	--
Female wound vrs female environment	0.689 (0.011)	.742 (.016)		--
Maternity wounds vrs theatre environment	0.824 (0.005	--	.862 (.015)	--
Maternity wounds vrs maternity ward environment	0.811 (0.042)	.234 (.342)	--	.996 (.002)

(-) = no correlation; OR=Odds ratio; P=p value. Close to (1) indicates correlation, Close to (-1) indicates correlation. ≤0.05 indicates significance.

proportion to erythromycin was 94.7%, but was 89.5% to tetracycline and penicillin. Resistant proportions to other antimicrobials were cefuroxime 78.9%, cotrimoxazole 73.7%, and gentamicin 21.10%. Resistance levels of other antibiotics are indicated in Table 8.

DISCUSSION

Surgical procedures have improved worldwide and deaths occurring from them have reduced considerably. Despite these advances, surgical site infections continue to be a major cause of morbidity and long hospital stay among these patients (de Lissovoy et al., 2009). This study was conducted with the aim of identifying the different types of bacteria contaminating post-operative wounds at the Edwin Cade Memorial Hospital, AngloGold Ashanti, Obuasi. Bacterial isolates from the wounds were compared with environmental contaminants isolated in the operating theatre and the surgical wards so as to determine whether the wound isolates were from the environment.

The study results indicate a high level of bacterial contamination of surgical wounds, with eight different bacterial types isolated. The theatre and surgical ward environments were found to be contaminated with bacteria similar to those isolated from the surgical wounds. The bacteria isolates from the wounds were probably from the environment since isolates from both sites (wounds and environments) were very much similar. The prevalence of bacterial infection of post-operative wounds in this study was 86%, a value considered high compared to 45% reported from India (Lilani et al., 2005), 34% reporded from Yemen (Nasser et al., 2013) and 57.4% reported from Mali (Togo et al., 2010) and 10,9% in Ethiopia (Wondimagegn et al., 2012). Bacteria types that contaminate surgical wounds vary with *S. aureus* and coagulase negative staphylococci which are frequently reported as the most common isolates (Anderson and Kaye, 2009; Anguzu and Olila, 2007). In the present study, Gram negative rods such as *E. coli* (16.3%), *P. mirabilis* (7.8%) and *P. aeruginosa* (6.2%) were also isolated. The degree of wound contamination has been linked to the kind of surgery performed. The kind of surgery has been classified variously to include: clean, clean- contaminated, contaminated and dirty, depending on the bacterial burden at the surgery site (Wassef et al., 2012). This classification scheme is used to predict surgical wound infection (Nguyen et al., 2001). The degree of contamination of clean surgery contamination is also influenced as described by Yi et al. (2011). Infections of Clean surgery site are determined by level of contamination of the theatre environment and the duration of the surgery (Pokrywka and Byers, 2013). The durations of the surgeries performed were not determined in this present study, but the environment was sampled

Table 8. Isolate proportions resistant to antibiotics (%).

Isolate	Tetracycline	Co-trimoxazole	Ampicillin	Gentamicin	Cefuroxime	Flucloxacillin	Erythromicin	Penicillin	Chloramphenicol	Cefotaxime	Ceftriaxone
S. aureus	90	94	100	84	100	92	48	100			
CNS	100	100	100	100	100	100	75	100			
K. pneumoniae	100	95	100	40	70		-		60	75	60
E. coli	92	71	100	54	73		-		67	63	58
P. mirabilis	75	100	100	100	100		-		100	100	100
P. aeruginosa	100	100	100	100	100		-		100	100	100

or bacteria. It was found that bacterial contamination was found in all the surgically related units, which are the surgical theatre and the surgical wards. These units have bacteria contaminants isolated from the air, door handles and bedsteads (Tables 5 and 6). Wound infections on females on the surgical ward recorded 92% wound infections and 81.4% on the maternity ward as compared to the 84.2% on the male surgical ward. This finding is in support of other findings that there is a higher risk of post-operative wound infections among females than males (Brandt et al., 2006; Manilich et al., 2013; Nasser et al., 2013).

S. aureus recording prevalence 57.9% was the most common isolate obtained from the surgical wounds and gain was the most common isolate obtained from almost all the surgical procedures performed. Furthermore, *Staphylococcus aureus* was found on many of the fomite types sampled from the environment. It is known that many healthy people carry *S. aureus* in their nostrils, skin, axilla and hairlines (Crawford et al., 2012) such carriers might shed the organism in the environment. The surgical wounds might have got infected from such contaminated environmental fomites or from such endogenous sources (Gottrup, 2005). It is for this reason that prophylactic antibiotic is given to reduce bacterial load at such sites prior to surgery (Crawford et al., 2012). Endogenous organisms such as *S. aureus* may not be cleared completely from the patient during the prophylactic cover prior to surgery and may re-colonize the surgical site soon after surgery (Anderson and Kaye, 2009). They therefore can cause nosocomial infection (Hawn, 2010), even if the patients are adequately given antibiotic prophylaxis before the surgery (Hirsch et al., 2010). Surgical site infections have been linked to environmental contamination in the surgical theatre and surgical wards (Adamina et al., 2013) as it was found in this present study that fomites sampled in the theatre had virtually no reduction in bacterial numbers after the theatre was cleaned and disinfected.

Organisms isolated from hospital environment might have had contact with several antibiotics (Aykan et al., 2013), so they are often multidrug resistant as a result of the production of antibiotic hydrolyzing enzymes such as the β-lactamases (Wasnik, 2013). Bacillus species remain unaffected by disinfectants and antimicrobials (Hirsch et al. 2010), because *Bacillus spp.* often survive in the environment in the form of spores which are unaffected by disinfectants. The door handles were frequently handled by patients, hospital workers and other people who enter the hospital facility making the ward door handles highly contaminated (Hota, 2004). In this study all the *S. aureus* isolated from contaminated surgical wounds were resistant to Penicillin and Ampicillin while over 50% were resistant to Erythromycin (Table 7). This observation is consistent with many other studies (Anderson and Kaye, 2009; Anguzu and Olila, 2007; Crawford et al., 2012) that *S. aureus* and enteric gram negative microbes have very high proportions being resistant to the commonly prescribed antimicrobials and tend to cause nosocomial infections.

The roles of organisms in the surgical theatre environment in contaminating surgical sites have been recognized in relation to failure of disinfectants and antibiotic prophylaxis. Attempts have therefore been made to reduce contamination of the surgical theatre by the use of laminar airflow in the theatre, but this gave no benefit and was associated with higher risk for severe post surgery wound infection (Brandt et al., 2008). There are many other factors that influence post surgery complications such as operative time, Body Mass Index (BMI), age, level of experience of the surgeon and the type of surgery performed (Manilich et al., 2013). Other factors that influence surgical site infection are preventive, and these involve skin preparation, wound closure technique adopted by the surgeon

and the most significant among them is the degree of microbial contamination of the surgical environment (Bode et al., 2010). These factors interplay with level of environmental contamination to determine frequency of surgical site infection (Brandt et al., 2008). In the light of these factors, extensive infection control practices are necessary to prevent pathogens invading surgical wounds to cause infection. The hospital is a place that keeps patients who are ill, and many of them are severly immunocompromised. A patient's immunity is breached by surgery so such a surgery patient needs to be protected, especially from surgical site infection. In conclusion, the results from this study at the Edwin Cade Memorial Hospital, AngloGold Ashanti, Obuasi showed that the prevalence of bacterial post-operative wounds infection was very high with a variety of microorganisms.

The organisms isolated from the wounds were similar to those isolated from theatre and surgical ward environments and they had similar antibiotic susceptibility patterns suggesting that the environmental isolates might be responsible for infecting the wound. Infection control measures in such critical areas such as the surgical theatre and surgical wards of the hospital need to be reviewed and strengthened.

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Vol. 8(15), pp. 1627-1632, 9 April, 2014
DOI: 10.5897/AJMR2013.6458
Article Number: DD754F243847
ISSN 1996-0808
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http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Biochemical and molecular identification of enteroaggregative *Escherichia coli* associated with childhood diarrhea and antimicrobial susceptibility profile of the isolates in Egypt

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Received 28 October, 2013; Accepted 24 March, 2014

Molecular identification and antimicrobial susceptibility of enteroaggregative *Escherichia coli* (EAEC) associated with childhood diarrhea was done out in Egypt. The usefulness of quantitative biofilm assay in detection of EAEC was compared with multiplex polymerase chain reaction (PCR). One hundred and fifty cases of childhood diarrhea were divided into three groups; 50 cases of acute diarrhea (group I), 50 cases of persistent diarrhea (group II) and 50 cases of healthy subjects of matched age and sex as a control group (group III). *E. coli* was isolated and identified by conventional microbiological methods. EAEC was detected by multiplex PCR and quantitative biofilm assay. Antimicrobial susceptibility profile of the isolated EAEC strains was done using disc diffusion method. *E. coli* was isolated from 78% (39/50) cases of acute diarrhea and 76% (38/50) cases of chronic diarrhea. The results show no significant difference between the results of multiplex PCR and quantitative biofilm assay; in 77 *E. coli* isolates, 15 (19.5 %) generated positive results for EAEC with multiplex PCR for two the specific genes AggR and EAST and 12 (15.6 %) strains showed positive results for EAEC by quantitative biofilm assay. As regard the antimicrobial susceptibility profile of the isolated EAEC strains, the results show that 85.7 and 87.5% of the EAEC strains isolated from cases of acute and persistent diarrhea, respectively were sensitive to amikacin, 47.1 and 62.5% were sensitive to cefoperazone, 28.5 and 50.00% were sensitive to ceftriaxone and 42.8% and 62.5% were sensitive to imepenem, 28.5 and 12.5 % of the EAEC strains isolated from cases of acute and persistent diarrhea respectively were sensitive to Amoxicillin-Clavulanic. All the isolated EAEC strains (100.00%) were resistant to sulphamethoxole/trimethoprim. High incidence of EAEC associated diarrhea among pediatric cases in Egypt must be considered before decision of antimicrobial therapy. Quantitative biofilm assay can be simple, rapid and convenient method for detection of EAEC in comparison with molecular methods and can therefore be recommended as a rapid screening test for EAEC in clinical laboratories.

Key words: Enteroaggregative *Escherichia coli* (EAEC), infantile diarrhea, multiplex polymerase chain reaction (PCR), quantitative biofilm.

INTRODUCTION

Enteroaggregative *Escherichia coli* (EAEC) have emerged as an important pathogen associated with endemic and epidemic diarrheal diseases in both industrialized and developing countries (Albert et al., 1999). In Egypt, about 16% of population is children under 5 years of age. Each child suffers, on the average, 3 bouts of acute diarrhea yearly, that is, 10 million children suffer 30 million episodes of acute diarrhea every year. Diarrhea accounts for 20-25% of deaths among children younger than five years. Diarrhea is a leading cause of under nutrition and poor growth, causing prolonged morbidity that may end fatally (El-Mougi 1999). EAEC strains are defined by their characteristic "stacked brick" aggregative adherence (AA) pattern to cultured epithelial cells (Nataro et al., 1992) and this is the basis of the assay considering the gold standard for EAEC identification. However, this technique requires specialized facilities and can therefore be performed only in reference laboratories. As alternative to this technique, a variety of phenotypic and molecular assays have been proposed (Wakimoto et al., 2004). Recently, a multiplex PCR assay for EAEC detection has been developed; one of these assays detects simultaneously three EAEC plasmid-borne genes: *aggR*, which encodes a central regulator involved in the expression of several EAEC virulence genes *(*Pass et al., *2000); aap*, which encodes the antiaggregation protein dispersin (Kimata et al., 2005) and *aatA*, which is part of a gene cluster that codes for a specific ATP-binding cassette transporter system (Sarantuya et al., 2004). These molecular techniques are of high costs and difficult to apply in clinical laboratories (Wakimoto et al., 2004; Sarantuya et al., 2004).

Thus, it is difficult to screen for EAEC among *E. coli* isolates from patients with diarrhea in clinical laboratories. The use of biofilm assays may be useful in overcoming these difficulties. Nataro and Kaper (1987) reported that EAEC produces a bacterial film on a polystyrene surface that could be easily visualized with Giemsa, a character which is used as a base for quantitative biofilm assay (Nataro and Kaper, 1987). The aim of this study was to evaluate the usefulness of the quantitative biofilm assay to screen the prevalence of EAEC among the clinical isolates causing acute and persistent diarrhea in pediatric cases and study the antimicrobial susceptibility profile of the isolated EAEC strains.

MATERIALS AND METHODS

After Research Ethical Committee approval and a written informed consent from parents of all participants in this research, this prospective randomized control study was conducted between 1/3/2012 to 1/3/2013 at Diarrhea and Malnutrition Unit in Pediatrics Department, Tanta University Hospital. The study was carried out on 150 cases divided into three groups: group I: 50 patients with persistent infantile diarrhea, group II: 50 patients with acute infantile diarrhea, group III: 50 healthy subjects of the same age group as a control group. Inclusion criteria: All infants suffering from acute or persistent diarrhea; Exclusion criteria: Antibiotic treatment for at least five days before this study, chronic disease and systemic infection.

Microbiological study (stool culture for isolation of *E. coli*)

Stool specimen were sent to microbiological laboratory as soon as possible for bacteriological study that include Gram stain smears to detect *E. coli* in stool specimens as Gram-negative bacilli, culture in aerobic facultative anaerobic incubator, in 37°C, for 24 - 48 h, on MacConkey's medium, and then the colonies were identified by biochemical reactions which include action on sugar media including lactose, sucrose, glucose, maltose, mannitol; action on triple sugar media and IMViC formula including (indole test, methyl red test, Voges proskaur test, citrate utilization test).

Multiplex PCR

The isolated strains of *E. coli* were also characterized by a multiplex PCR with below mentioned primers for the detection of two specific genes *aggR* (630 bp) and *east* (97 bp). The primers were chosen from a reference protocol (Kahali et al., 2004). For standardization purpose we used positive 042 strain and 044 strains as control strains.

Bacterial lysates were prepared by re-suspending a single colony in 1 ml of deionized water in a sterile 5 ml glass tube followed by boiling for 10 min at 95°C. After boiling the suspension is centrifuged at 10,000 rpm for 10 min and the supernatant solution is directly used as a template for PCR.

Each PCR tube contained 50 µl of reaction mix [(10x PCR buffer with $MgCl_2$; dNTP mix 2.5 mM each; 4 primers 10 mM each, which comprised of *aggR* 5' CTGGCGAAAGACTGTATCAT' 3 + 5' CAATGTATAGAAATCCGCTGTT' 3 and for *east* 5' CACAGTATATCCGAAGGC' 3 + 5' CGAGTGACGGCTTTGTAG' 3, Template lysate, sterile water, Taq polymerase (5U/l)] and total volume made up to 50 µl.

The solutions were then subjected to the following cycling conditions- denaturation 94°C/1 min, annealing 55 °C/1 min, extension 72°C/1 min, final extension 72° C/7 min in a thermal cycler. Then 10 µl of the PCR mixture was visualized by ethidium bromide staining after electrophoresis in 2% agarose gel in tris acetate -EDTA buffer.

Quantitative biofilm assay

To assess biofilm formation, we inoculated 200 µL of Dulbecco's modified Eagle's medium containing 0.45% glucose in 96-well flat-bottom microtiter polystyrene plates (Becton Dickinson, Franklin

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Table 1. Demographic and clinical characteristics of the studied groups in relation to EAEC infection.

Demographic and clinical characteristics			Quantitative biofilm assay			Chi-square	
			Negative to EAEC	Positive to EAEC	Total	X^2	P-value
Age	<2years	N (%)	60 (70.6)	12 (80.0)	72		
	>2years	N (%)	25 (29.4)	3 (20.0)	28	0.447	0.896
sex	Female	N (%)	41 (48.2)	7 (46.7)	48		
	Male	N (%)	44 (85.0)	8 (53.3)	52	0.523	0.774
Resident	Urban	N (%)	21 (24.7)	3 (20.0)	24		
	Rural	N (%)	64 (75.3)	12 (80.0)	76	1.336	0.241
Season	Summer and autumn	N (%)	60 (70.6)	9 (60.0)	69	1.447	0.335
	Winter and spring	N (%)	25 (29.4)	6 (40.0)	31		
Feeding pattern	Breast feeding	N (%)	53 (62.4)	4 (26.7)	57	12.336	0.001*
	Non breast feeding	N (%)	32 (37.7)	11 (73.3)	43		
Vomiting	Negative	N (%)	29 (34.1)	5 (33.3)	34	0.420	0.361
	Positive	N (%)	56 (65.9)	10 (66.7)	66		
Dehydration	No dehydration	N (%)	45 (52.9)	6 (40.0)	51	1.632	0.147
	Some dehydration	N (%)	34 (40.0)	9 (60.0)	43	1.985	0.256
	Severe dehydration	N (%)	6 (7.1)	0 (0.0)	6	1.996	0.062
Fever	Low	N (%)	50 (58.8)	9 (60.0)	59	1.669	0.255
	High	N (%)	35 (41.2)	6 (40.0)	41		
Mucus	Negative	N (%)	58 (68.2)	1 (6.7)	59	14.668	0.001*
	Positive	N (%)	27 (31.8)	14 (93.3)	41		
RBCs	Negative	N (%)	66 (77.7)	4 (26.7)	70	15.575	0.001*
	Positive	N (%)	19 (22.4)	11 (73.3)	30		
Pus	Negative	N (%)	33 (38.8)	7 (46.7)	40	1.574	0.225
	Positive	N (%)	52 (85.0)	8 (15.0)	60		

*Significant at P-value < 0.05.

Lakes, NJ) with 5 µL of an overnight Luria broth culture grown at 37°C with shaking. The sample was incubated overnight (18 hours) at 37°C and visualized by staining with 0.5% crystal violet for five minutes after washing with water. The biofilm was quantified after adding 200 µL of 95% ethanol, by an enzyme-linked immunosorbent assay plate reader at 570 nm. Strain EAEC 042 was used as a positive control and *E. coli* HB101 was used as a negative control. All EAEC strains showed absorbance >0.2 (Sarantuya et al., 2004).

Antibiotic sensitivity test

Antibiotic susceptibility testing of EAEC isolates was performed using the standardized disc agar diffusion method (Oxoid-England) using discs of Cefebime (30 µg), Amikacin (30 µg), Co-trimoxazol (25 µg), Ciprofloxacin (5 µg), Imipenem (10 µg), Amoxillin-clavulanic (10 µg) and Cefotriaxone, Cefoperazone (10µg). Interpretation of the results was done according to CLSI guidelines 2008.

Statistics

Statistical presentation and analysis of the present study was conducted, using Chi-square test by SPSS V.16.

RESULTS

The present work was carried out on fifty children suffering from acute diarrhea, their age ranged between 2 months and 6 years (mean ± SD: 2 ± 3.54), they were 27 males and 23 females and another fifty children suffering from persistent diarrhea were used, their age ranged between 2 months and 4 years (mean ± SD: 2 ± 5.14), they were 30 males and 20 females. All cases were attending Diarrhea and Malnutrition Unit in Pediatrics Department, Tanta University Hospital. Fifty normal healthy children of matched age and sex served as a control group. Demographic and clinical characteristics of the studied groups in relation to EAEC infection are presented in Table 1.

The results of this study show that *E. coli* represent the causative organism of infantile diarrhea in 39 out of 50 cases of acute infantile diarrhea (39%) and 38 out of 50 cases of persistent infantile diarrhea (38 %). None of the 50 control samples collected showed positive results with biofilm nor generated positive PCR for two specific genes tested.

The results showed that out of the total 77 *E. coli*

Table 2. Comparison between the result of quantitative biofilm assay test and multiplex PCR with regards to EAEC infection.

	Positive		Negative		Total		Chi-Square	
	N	%	N	%	N	%	X^2	P-value
Quantitative biofilm assay	12	12	88	88	100	100		
Multiplex PCR	15	15	85	85	100	100	0.168	0.682

Table 3. Antimicrobial susceptibility pattern of EAEC isolates from cases of acute diarrhea.

Antimicrobial agent	Resistant N (%)	Highly sensitive N (%)	Moderately sensitive N (%)
Amikacin	0 (0.0)	6 (85.7)	1 (14.2)
Amoxicillin-Clavulanic	5 (71.4)	0 (0.0)	2 (28.5)
Cefoperazone	(0.0)	4 (47.1)	3 (42.8)
Cefotrioxone	2 (28.5)	2 (28.5)	3 (42.8)
Ciprofloxacin	4 (47.1)	2 (28.5)	1 (14.2)
Cefibim	4 (47.1)	0 (0.0)	3 (42.8)
Sulphamethole/Trimethoprim	7 (100.0)	0 (0.0)	0 (0.0)
Imepenem	2 (28.5)	3 (42.8)	2 (28.5)

Table 4. Antimicrobial susceptibility pattern of EAEC isolates from cases with persistent infantile diarrhea.

Antimicrobial agent	Resistant N (%)	Highly sensitive N (%)	Moderately sensitive N (%)
Amikacin	0 (0.0)	7 (87.5)	1 (12.5)
Amoxicillin-Clavulanic	7 (87.5)	0 (0.0)	1 (12.5)
Cefoperazone	0 (0.0)	5 (62.5)	3 (37.5)
Cefotrioxone	2 (25.0)	4 (50.0)	2 (25.0)
Ciprofloxacin	5 (62.5)	3 (37.5)	0 (0.0)
Cefibim	7 (87.5)	0 (0.0)	1 (12.5)
Sulphamethole/Trimethoprim	8 (100.0)	0 (0.0)	(0.0)
Imepenem	3 (37.5)	(0.0)	5 (62.5)

isolates, 15 generated positive results with multiplex PCR for two specific genes *aggR* and *east*. By quantitative biofilm assay, 12 (80 %) strains showed positive results by Quantitative microtitre plate assay (P-value 0.682) (Table 2).

As regard the antimicrobial susceptibility profile of the isolated EAEC strains the results showed that 85.7 and 87.5% of the EAEC strains isolated from cases of acute and persistent diarrhea respectively were sensitive to Amikacin, 47.1 and 62.5% were sensitive to cefoperazone, 28.5 and 50.00% were sensitive to Ceftriaxone and 42.8 and 62.5% were sensitive to Imepenem. 28.5 and 12.5% of the EAEC strains isolated from cases of acute and persistent diarrhea respectively were sensitive to Amoxicillin-Clavulanic. All the isolated EAEC strains (100.00%) were resistant to Sulphamethoxole/Trimethoprim (Tables 3 and 4).

DISCUSSION

The importance of EAEC strains in public health around the world is becoming increasingly clear. The EAEC strains have been associated classically with persistent diarrhea (≥ 14 days) and with growth retardation in infants (Iwanaga et al., 2002). EAEC diarrhea involves bacterial aggregation, adherence to intestinal epithelial cells and elaboration of several toxigenic bacterial mediators. EAEC is primarily recognized as a cause of endemic and persistent childhood diarrhea in developing countries (Gascon et al.,

2000; Albert et al., 1999). Therefore, the detection of EAEC strains can make a significant contribution to public health in many areas. The present work was carried out on fifty children suffering from acute diarrhea their age ranged between two months and six years, they were 27 males and 23 females and fifty children suffering from persistent diarrhea their age ranged between two months to four years, they were 30 males and 20 females. All cases were admitted to Diarrhea and Malnutrition Unit in Pediatrics Department, Tanta University Hospital. Fifty normal healthy children of matched age and sex served as the control group. The results of this study showed that *E. coli* represent the causative organism of diarrhea in 39 out of 50 cases of acute infantile diarrhea (39%) and 38 out of 50 cases of persistent infantile diarrhea (38%).

Of the total 77 *E. coli* isolates, 15 generated positive results with multiplex PCR for two specific genes *aggR* and east. When these 15 PCR positive strains were studied for biofilm production, 12 (80%) strains showed positive results by quantitative microtitre plate assay. Raju and Ballal (2007) reported that of the total 100 *E. coli* isolates, 23 generated positive results with multiplex PCR for two specific genes *aggR* and *east*. Of which 20 (86%) strains showed positive results by quantitative microtitre plate assay. They also found that none of the 50 control samples collected showed positive results with biofilm. On the other hand, Helmi et al. (2010) showed that by quantitative biofilm assay out of total 300 *E. coli* isolates (200 cases and 100 controls) they could detect 65 EAEC strains (32.5 %). All controls showed negative results. This discrepancy may be attributed to different antimicrobial policy used in each community.

The results of this study showed that EAEC were detected in all age groups, especially in the less than 2 year age group; 12 cases (80%) less than two year, three cases (20%) more than 2 year. In accordance with these results, Helmi et al. (2010) showed that fifty five (84.5%) out of the 65 EAEC strains were isolated from patients below 24 months of age (15). The study also was in agreement with the study of Lima et al. (2000). Almost all patients (86%, positive EAEC) were under 24 months of age, suggesting the development of resistance against agents could be with increasing age.

The results of the study show that there was no significant difference in the presence of EAEC in both sexes, between rural and urban areas. Also, there was no significant difference in seasonal presence between EAEC positive and negative cases. On contrary to our results, Helmi et al. (2010) showed that the presence of diarrhea showed higher rates which was recorded in the months of June-August, than in the months of December-February. This might be attributable to the fact that most common bacterial pathogens causing acute diarrhea occur during summer, while most common viral pathogens occur during winter.

In the present study, EAEC detection rates were higher in infants that were not breastfed. Exclusive breast feeding was found to be significantly associated with lower presence of EAEC diarrhea. This observation was supported by the study of Ghosh et al. (2001) where 7 of the 109 infants harboring EAEC were breastfed, while the remaining 102 were on other feeding modes.

In this study, the infection with EAEC strains is associated with watery mucoid, bloody diarrhea, low grade fever and sometimes vomiting. The presence of fever, vomiting, dehydration or pus in stools did not differ significantly between EAEC positive and negative cases. The study of Helmi et al. (2010) showed that the nature of diarrhea was watery mucoid in 76.9%, versus bloody mucoid in 23.1% of patients. Adachi et al. (2002) stated that the clinical symptoms of EAEC infection vary from one study to another. Although not all EAEC infections result in symptomatic illness, most studies suggest that EAEC infection results in gastrointestinal disease. The most commonly reported symptoms are watery diarrhea with or without blood and mucus, abdominal pain, nausea, vomiting, and low grade fever. EAEC can cause both an acute and a persistent (>14 days) diarrheal illness. EAEC is associated with significant fluid loss and dehydration but a bloody stool is relatively infrequent Fran et al. (2011).

As regard the antimicrobial susceptibility profile of EAEC strains, the results of this study showed that the highest sensitivity of the isolated strains was to Amikacin (85.7 and 87.5%) and Cefoperazone (47.1 and 62.5%) then to Cefotrioxone (28.5 and 50.00%) and Imepenem (42.8 and 62.5%) in acute and persistent diarrhea and the lowest sensitivity was to sulphamethoxole/-trimethoprim (0.0 %) and Amoxicillin-Clavulanic (28.5 and 12.5%) in acute and persistent diarrhea. In accordance with these results, Paterson and Yu (1999) showed that the highest sensitivity of the strains of EAEC was to amikacin and ceftazidime and the lowest sensitivity was to ampicillin. Glandt et al. (1999) showed that EAEC-mediated diarrhea responded to therapy with ciprofloxacin and this was actually supported by the dissimilarities in the intestinal inflammatory markers seen in the ciprofloxacin- and placebo-treated populations. In another study, Sang et al. (1997) studied the association of multi-drug resistant EAEC isolated from persistent diarrhea in Kenyan children, and they found that EAEC was resistant to tetracycline, ampicillin, erythromycin, trimethoprim-sulphamethoxazole and amoxillin /-clavulanate. These discrepancies of the results of antimicrobial susceptibility profile of EAEC in the different studies may be due to the different policies of antibiotic therapy in different communities and this may be alarming for the importance of rapid and economic detection of EAEC in childhood diarrhea to improve the morbidity and mortality of the disease.

On another side, Sobieszczanska et al. (2003) showed

that many EAEC infections are self-limited. Symptomatic infections are usually treated empirically because laboratory diagnosis is not routinely available. EAEC susceptibility varies by region. In most regions, EAEC strains are susceptible to the fluoroquinolones, azithromycin, rifaximin, amoxicillin/clavulanic acid and nalidixic acid.

Conclusions

High incidence of EAEC associated diarrhea among pediatric cases in Egypt must be considered before decision of antimicrobial therapy. Quantitative biofilm assay is simple, rapid and convenient method for detection of EAEC in comparison with molecular methods and can therefore be recommended as a rapid screening test for EAEC in clinical laboratories.

Conflict of Interests

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

ACKNOWLEDGEMENTS

The authors greatly appreciate all members of Medical Microbiology and Immunology Department, and Pediatric Department, Faculty of Medicine, Tanta University, Tanta, Egypt for their great support and help.

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Vol. 8(15), pp. 1633-1637, 9 April, 2014
DOI: 10.5897/AJMR2013.5779
Article Number: 93A61B443856
ISSN 1996-0808
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http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Microbiological assessment of *kunun-zaki* marketedin Abuja Municipal Area Council (AMAC) in The Federal Capital Territory (FCT), Nigeria

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Received 29 March, 2013; Accepted 20 January, 2014

Forty-one (29) *kunun-zaki* samples were obtained as freshly formulated beverages from different local hawkers in 10 different locations in Abuja Municipal Area Council (AMAC) of the Federal Capital Territory (FCT) Abuja, Nigeria. The samples were evaluated for bacterial loads, isolation and identification of microorganisms present using spread plate agar dilution method. Bacterial loads ranged from 0.0 to 2.0 × 10^8 CFU/mL. The pH ranged between 2.64 to 5.0. The microorganisms isolated were identified by biochemical tests and microscopic analysis. The organisms isolated from the samples include *Staphylococcus aureus*, *Klebsiella* spp., *Eschericha coli*, *Citrobacter* spp., *Salmonella typhi*, *Shigella* spp., *Candida albicans*, *Lactobacillus* spp., *Aspergillus fumigatus*, *Aspergillus niger*, *Penicillium* spp. and *Saccharomyces cerevisiae.* The high bacterial load of most of the samples can be attributed to the poor hygienic practices of the handlers and possible contamination from the utensils and water that were used for processing of the beverage. The presence of these organisms could be a matter of serious concern as these organisms are involved in some health implications causing various diseases.

Key words: *Kunun-zaki*, spread plate, microbial contamination, Abuja.

INTRODUCTION

Kunun-zaki is a traditional fermented non-alcoholic beverage widely consumed in Northern Nigeria. It is widely consumed for its thirst quenching properties most especially during the dry season (Elmahmood and Doughari, 2007). It can be produced from millet, sorghum or maize. It has immense social, economic, nutritional and medicinal benefits to numerous consumers. *Kunun-zaki*, like other locally made drinks is widely consumed in Nigeria. In most Nigerian cities, the sales and consumption of this locally made beverage is high due to the high cost of other non-alcoholic drinks. Due to the non-alcoholic nature of this drink, it is widely accepted and consumed by both Muslims and Christians alike as a substitute for alcoholic drinks. The drink is usually sold at the motor parks, school premises and market places and even served during social gatherings (Abegaz, 2007).

Production methods are crude, ingredient concentrations are neither quantified nor standardized, instead preparation is largely a matter of family tradition (Onuorah et al., 1987). Significant variations exist in the production

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procedures depending on its taste and cultural habits of its consumers leading to differences in quality and stability. The high water content (about 85%) coupled with crude methods of production and packaging under inadequate sanitary conditions predisposes *kunun-zaki* to microbial contamination (Elmahmood and Doughari, 2007; Ayo et al., 2010). Osuntogun and Aboaba (2004) reported that *kunun-zaki* is prone to microbial deterioration if not adequately stored. *Kunun zaki* has been reported to have a shelf life of about 24 h (Adeyemi and Umar, 1999). The occurrence of wide genera of micro-organisms could be attributed to the unhygienic conditions of preparation and the use of contaminated raw materials and utensils (Ayo et al., 2004). The micro flora of the finished product depends on the processing and storage conditions. High temperature and lack of refrigeration facilities in most developing countries have led to the inability to produce and store fresh *kunun-zaki*.

This study was designed to assess the microbial quality of this indigenous beverage in the Abuja Municipal Area Council (AMAC) region of The FCT, Abuja, Nigeria and possibly highlight the risks involved in the consumption by the general public as well as the possible source of contamination.

MATERIALS AND METHODS

Collection of samples

Samples of freshly prepared *kunun-zaki* were collected from different hawkers from 10 different locations within the Abuja Municipal Area Council (AMAC) FCT, Abuja, Nigeria. The samples were labeled and transferred within 3 h of collection to the microbiology laboratory in their original package and the contents aseptically withdrawn from the bottles for isolation of micro-organisms and enumeration of bacteria.

Determination of pH of the samples

The pH of the various samples was within 3h of collection determined using sterile probes (disinfected with alcohol) of the pH meter (Corning 35).

Determination of total count of bacteria

Bacterial total count was carried out on plates of Nutrient agar (NA, Oxoid), using the spread plate method. The samples were serially diluted up to 10^6 ml and 0.1 ml of appropriate dilution was used to inoculate each of the plates in duplicates. The culture plates were then incubated at 37ºC for 48 h and colonies counted manually. The mean of triplicate results was recorded as the colony count (Oshoma et al., 2009).

Isolation and Identification

Discrete colonies of the organisms isolated (for bacteria) were selected and sub cultured from the plates to respective nutrient agar plates and incubated at 37ºC for 24 h. The bacterial isolates were identified following standard microbiological procedures as described by Buchanan and Gibbons (1974) and Cheesbrough (2002). For the filamentous fungi, appropriate spore dilutions (1.0 × 10^7 spores/ml) of the fungal isolates were surface-spread in duplicates on Sabouraud Dextrose Agar (SDA, Oxoid) plates and incubated at room temperature (25 to 27ºC) for 48 to- 72 h. The colonies were screened and identified based on the taxonomic schemes and descriptions by Ainsworth et al. (1973) and Mislivec et al. (1992).

RESULTS

Mean pH and total viable counts

The mean pH values and level of microbial contamination is shown in Table 1. The *kunun-zaki* samples had a pH range of 2.64 to 5.0. All samples were acidic in nature. Sample Kw3 had the lowest pH of 2.64 while sample Kw1 had the highest pH of 5.0. The bacteria count ranged from 0.0 to 2.0 × 10^8 cfu/ml. Samples A had highest bacteria count while samples E had the lowest bacteria count.

Table 2 shows the different microorganisms isolated from the *kunun-zaki* samples. *Saccharomyces cerevisiae* dominated the organisms as it was isolated from most of the samples (34.15%). This was closely followed by *Klebsiella* sp. isolated from nine samples (21.95%). *Staphylococcus aureus* was isolated from six samples (14.64%) and *Aspergillus niger* from five samples (12.2%). Other microbes isolated from the *kunun-zaki* samples include *Escherichia coli Candida albicans* and *Aspergillus fumigatus* isolated from two samples each (4.88%). *Citrobacter* spp., *Salmonella typhi, Shigella* spp., *Lactobacillus fermentum* and *Penicillium* spp. were isolated from one sample each (1.44%).

Cultural characteristics and biochemical Identification of isolated strains

A total of 22 bacterial types were isolated. Non-red colonies from MacConkey plates that grew with golden yellow on mannitol salt agar and were Gram positive, coagulase-positive and catalase-positive were taken as *Staphylococcus aureus*; non-red colonies from MacConkey plates that were Gram-negative, indole-negative, methyl red-positive, Voges-Proskauer-negative, citrate-negative, acidic butt, alkaline slant with no blackening on TSI slant were taken as *Shigella* spp., non-red colonies from MacConkey plates that were Gram negative, indole-negative, methyl red-positive, Voges-Proskauer-negative, citrate-positive, acidic butt and alkaline slant with blackening on TSI slant, urease-negative and colourless colonies with black center on SS agar were taken as *Salmonella* spp., Red colonies from MacConkey plates that grew with greenish metallic sheen on eosin methylene blue agar and were Gram negative, indole-positive, methyl red-positive, Voges-Proskauer-negative and citrate-negative were taken as *E. coli*; red mucoid colonies from MacConkey plates that were Gram negative, indole-negative, methyl red-negative, Voges-Proskauer-positive and citrate-positive were taken as *Klebsiella* spp.; non spore forming Gram positive,

Table 1. Mean pH values and Total viable counts (cfu/ml) for fresh *kunun-zaki*.

Location	Sampling no.	Average pH	Total viable count (cfu/mL)
Idu	Id1	3.55 ± 0.0	$8.4 \times 10^{5} \pm 0.0$
	Id2	3.24 ± 0.04	$8.4 \times 10^{7} \pm 0.0$
	Id3	3.31 ± 0.03	$5.0 \times 10^{6} \pm 0.0$
	Id4	3.13 ± 0.01	$2.2 \times 10^{5} \pm 0.58$
Lugbe	Lu1	3.55 ± 0.0	$3.6 \times 10^{3} \pm 0.0$
	Lu2	3.02 ± 0.02	$2.9 \times 10^{6} \pm 0.33$
	Lu3	3.52 ± 0.04	$2.0 \times 10^{8} \pm 0.0$
Garki	Gk1	3.40 ± 0.01	$1.0 \times 10^{5} \pm 0.0$
	Gk2	3.98 ± 0.01	$6.0 \times 10^{5} \pm 0.0$
	Gk3	3.92 ± 0.06	$2.0 \times 10^{5} \pm 0.0$
	Gk4	4.33 ± 0.17	$4.3 \times 10^{5} \pm 0.33$
	Gk5	4.02 ± 0.07	$2.0 \times 10^{5} \pm 0.0$
Wuse	Ws1	3.64 ± 0.0	$2. \times 10^{3}0 \pm 0.0$
	Ws2	4.20 ± 0.01	$1.2 \times 10^{7} \pm 0.58$
	Ws3	3.49 ± 0.01	$8.5 \times 10^{6} \pm 0.0$
	Ws4	4.22 ± 0.03	$8.4 \times 10^{5} \pm 0.0$
	Ws5	3.40 ± 0.025	$6.4 \times 10^{6} \pm 0.58$
Asokoro	As1	4.85 ± 0.02	$3.6 \times 10^{2} \pm 0.0$
	As2	2.94 ± 0.06	$1.1 \times 10^{8} \pm 0.33$
	As3	3.90 ± 0.01	$7.3 \times 10^{5} \pm 0.33$
	As4	3.32 ± 0.04	$2.1 \times 10^{6} \pm 0.58$
Mararaba	Mb1	4.50 ± 0.0	$5.1 \pm 0.33 \times 10^{2}$
	Mb2	3.17 ± 0.01	0 ± 0.0
	Mb3	3.11 ± 0.03	0 ± 0.0
	Mb4	3.13 ± 0.1	$1.0 \times 10^{3} \pm 0.0$
	Mb5	3.10 ± 0.01	0 ± 0.0
Kubwa	Kw1	5.0 ± 0.0	$3.0 \times 10^{3} \pm 0.0$
	Kw2	3.78 ± 0.06	$1.0 \times 10^{8} \pm 0.33$
	Kw3	2.64 ± 0.17	$5.0 \times 10^{6} \pm 0.0$
	Kw4	3.14 ± 0.01	$1.1 \times 10^{7} \pm 0.0$
Maitama	Mt1	4.55 ±0.01	$1.2 \times 10^{3} \pm 0.0$
	Mt2	3.08 ± 0.03	$6.1 \times 10^{7} \pm 0.0$
	Mt3	3.56 ± 0.08	$2.0 \times 10^{6} \pm 0.0$
	Mt4	3.19 ± 0.03	$1.2 \times 10^{5} \pm 0.0$
Jikwoi	Jw1	4.26 ± 0.0	$3.7 \pm 0.0 \times 10^{3}$
	Jw2	3.60 ± 0.01	$2.2 \pm 0.33 \times 10^{7}$
	Jw3	3.59 ± 0.05	$8.4 \pm 0.0 \times 10^{5}$
Karmo	Kr1	3.60 ± 0.0	$5.5 \pm 0.0 \times 10^{3}$
	Kr2	2.73 ± 0.056	$1.0 \pm 1.0 \times 10^{7}$
	Kr3	2.96 ± 0.04	$5.0 \pm 0.58 \times 10^{6}$
	Kr4	4.03 ± 0.01	$4.0 \pm 0.0 \times 10^{7}$

Table 2. Presence of Bacterial and Fungal Flora in *Kunu zaki* samples.

No. of sample	*Klebsiella* spp.	*Escherica coli*	*Staphylococcus aureus*	*Citrobacter* spp.	*Salmonella typhi*	*Shigella* spp.	*Candida albicans*	*Aspergillus Niger*	*Aspergillus fumigatus*	*Saccharomyces cerevisiea*	*Lactobacillus* spp.	*Penicillum* spp.
41	9	2	6	1	1	1	2	5	2	14	1	1
%	22.0	4.9	14.6	2.4	2.4	2.4	4.9	12.20	4.9	34.2	2.4	2.4

filamentous bacilli, catalase negative, glucose and mannitol fermenter were taken as *Lactobacillus* sp.; round, smooth , opaque colonies with regular margins from blood agar plates, Gram negative motile rods, indole-negative, methyl red-positive, Voges-Proskauer-negative and citrate-positive, urease positive, H_2S production on TSI agar slants, glucose, maltose and mannitol fermenter were taken as *Citrobacter* spp.; moist, creamy Gram positive colonies, germ tube test positive, urease negative, true and pseudo hyphae present, positive for assimilation of glucose, maltose, sucrose, galactose and negative lactose assimilation, glucose, maltose, galactose fermenter were taken as *C. albicans*. White to cream, smooth, glabrous yeast-like colonies on SDA plates, with large globose to ellipsoidal budding yeast-like cells or blastoconidia were taken as *S. cerevisiae* A compact white or yellow basal felt covered by a dense layer of dark-brown to black conidial heads on Czapek Dox agar, large, biserate dark brown to black conidia heads, globose, becoming radiate with the phialides borne on septate metulae which are twice as long as phialides were taken as *A. niger*. Blue-green surface pigmentation with suede like surface consisting of dense conidiophores on Czapek Dox agar, uniseriate and columnar conidial heads with the phialides limited to the upper two thirds of the vesicle and curving to be roughly parallel to each other were taken as *A. fumigatus*. Fast growing colonies in shades of green mostly consisting of a dense felt of conidiophores, globose, ellipsoidal, cylindrical, hyaline, smooth walled were taken as *Penicillum* spp.

DISCUSSION

All *kunun-zaki* samples were acidic in nature with pH range of 2.64 to 5.0. Various researchers have attributed this to the presence of fermentative microorganisms in *kunun-zaki* which cause spoilage of the beverage by fermentation of its carbohydrate content producing undesirable changes in them, altering their aroma and taste and thus making them unpalatable for human consumption. Osuntogun and Aboaba (2004) isolated lactic acid bacteria such as *Lactobacilli*, *Leuconostoc* and *Streptococcus* which were reported to possess the ability to ferment carbohydrates to produce lactic acid thus lowering the pH. *Lactobacilli* have also been isolated from other indigenous non- alcoholic beverage like *zoborodo*. The high bacteria load (5.1×10^2 to 2.0×10^8) of all the *kunun-zaki* samples can be attributed to the poor hygiene practices of the handlers and possible contamination from the utensils and water used for processing the beverage as well as the packages used in its distribution. The presence of *S. aureus, E. coli, Klebsiella* sp, *E. coli, S. typhi* and *Shigella* spp. could be a matter of serious concern, since these organisms are involved in some health implications.

Klebsiella is a gram negative bacilli belonging to the family Enterobacterioceae. It is usually associated with faecal contamination. Being an enteric bacterium its presence indicates poor hygiene practices among handlers. Due to the significance of the faecal-oral route transmission for many bacterial food-borne diseases, basic hygiene measures assume a decisive importance in food safety management (Uzeh et al., 2006).

S. aureus is a normal flora of the skin, nose, mucous membrane, throat, palms, hairs and a common etiological agent of septic arthritis. It is an ubiquitous microorganism that can enter foods from many sources such as handlers with acute pyogenic infections or healthy carriers who harbour the organism in their nose or throat. It is commonly implicated in water and food contamination. The detection of *S. aureus* is of serious public health importance because of its ability to cause a wide range of infections especially food-borne intoxication. This organism was equally isolated by Olasupo et al. (2002) from wara and *kunun-zaki*, a cereal based, non-alcoholic beverage.

E. coli is an important member of the coliform group. It is part of the normal flora of the human intestine. Some strains can cause gastroenteritis, diarrhoae and urinary tract infection. The presence of this organism in *kunun-zaki* is an indication of faecal contamination.

S. typhi an enteric bacteria is the causative agent of typhoid fever. The increased frequency of food-borne *Salmonella* has been causing recurring outbreaks, sometime with fatal infections which has been linked to the unsanitary practices of food and beverages processes leading to contamination of foods by *Salmonella* (Radji et al., 2010). The routine

detection of Salmonella in the environment including in foods and beverages is a necessary component of public health programs. The presence of *L. fermentum* and *S. cerevisiae* isolates in the samples analyzed is not as surprising as these organisms have been reported to thrive in medium rich in fermentable substrates. *Lactobacillus is* not usually pathogenic and is a known intestinal flora in humans. It has been reported to possess beneficial properties. These include colon cancer prevention, immune system enhancement and allergy reduction, owing to their ability to antagonize the activities of some food spoilage pathogenic bacteria like *S. aureus* and *E. coli* (Osuntogun and Aboaba, 2004). *S. cerevisiae* has been implicated in food spoilage due to its fermentative ability, osmophilic nature, tolerance of acid, tolerance of alcohol and ability to grow at low temperature (Badua, 2006).

Aspergillus and *Penicillium* species have also been implicated in food spoilage especially those with carbohydrate substrate. They are storage microflora of many cereals. Their growth can result in production and accumulation of mycotoxins which are of public health and economic importance (Rhodes and Flecher, 1966).

Conclusion

The presence of these isolated organisms in *kunun-zaki* samples analyzed could serve as indicator for the need to promote awareness about the possible health hazards that could arise due to handling and processing of the beverage. The range of microorganism isolated pose serious threat to food safety and hence the need to ensure microbial safety during the production and distribution of this drink that is widely consumed in most parts of Northern Nigeria.

ACKNOWLEDGEMENT

The authors wish to thank the DG/CEO of NIPRD Prof. K. S. Gamaniel and Staff of the Department of Microbiology and Biotechnology, NIPRD for their support.

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