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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

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

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

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African Journal of Biotechnology

Full Length Research Paper

Polymorphism, sequencing and phylogenetic characterization of growth differentiation factor 9 (*GDF9*) gene in Assam Hill goat

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Assam hill goat (*Capra hircus*) is a prolific local goat in India. Growth differentiation factor (*GDF9*) gene was studied as a candidate gene for the prolificacy of goats. The objective of the present study was to detect the incidence of mutation in the exonic region of *GDF9* gene of Assam hill goat. Total number of 90 blood samples were collected randomly from different parts of Assam and genomic DNA were extracted using modified phenol-chloroform method. The quantity and quality of extracted DNA was examined using spectrophotometry and gel electrophoresis, respectively. A 294 bp fragment of GDF9 gene was amplified using polymerase chain reaction (PCR). The purified product was digested with *Dde*I, *Hha*I and *Alu*I restriction enzymes which produced single type of banding pattern 242 and 52 bp, 140 and 154 bp, 134 and 160bp, respectively. The present study revealed wild type alleles and all the samples showed AA genotype. Nucleotide sequencing revealed two new mutations 495 (C → A) and 387 (G → A). Phylogenetic analysis showed that the sequences of Assam Hill goat belong to a common cluster which differs from that of the other goat breeds. The analysis of polymorphism for *GDF9* in Assam Hill goat indicates that the genetic factor responsible for prolificacy or multiple kidding rate is not related to the reported mutated alleles of *GDF9 gene.* Therefore, attempts to be made to detect other single nucleotide polymorphism (SNPs) for *GDF9* gene or otherwise effort should be made towards other fecundity gene which might be responsible for the prolificacy of Assam Hill goat.

Key words: Assam Hill goat, GDF9, polymorphism, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

INTRODUCTION

Growth differentiation factor 9 (*GDF9*) belongs to the transforming growth factor-b superfamily. In female reproduction, growth and differentiation factor plays a critical role during early folliculogenesis in mammals (Elvin et al., 1999). The *GDF9* gene in sheep has been mapped to chromosome 5 between the BM7247 and BMS2258 markers (Sadighi et al., 2002). The expression of *GDF9* was oocyte-specific in ovine and bovine ovaries which begin at the primordial follicle stage (Bodensteiner et al., 1999). *GDF9* mRNA and protein were expressed in

Table 1. Primers (Forward and reverse) along with the properties for amplification of 294 bp of GDF9 gene.

Primer	Primer sequence (5'-3')	Molecular weight	Tm (°C)	Length (mer)	GC content (%)	Size of amplicon (bp)	Reference
Forward	GTTGGAATCTGAGGCTGAG	5923.9	54.18	19	52.6	294	Chu et al., 2011
Reverse	ATCTGCTCCTACACACCTG	5683.7	51.83	19	52.6		

follicles caprine ovary at all stages of their development, and additionally in luteal tissue (Silva et al., 2004). *GDF9* gene mutations in sheep may cause either an increased ovulation rate or infertility. In sheep, four different mutations of *GDF9* had been identified which affect fertility including FecGH (G8) mutation in Cambridge and Belclare sheep (Hanrahan et al., 2004), in Santa Ines sheep FecGE or FecGSI mutation (Melo et al., 2008; Silva et al., 2010), FecTT mutation in Thoka sheep (Nicol et al., 2009). In Moghani and Ghezel sheep (Barzegari et al., 2010) and in Garole sheep (Polley et al., 2010), G1 mutation was found.

Genetic mutation of *GDF9* identified in sheep which showed major effect on ovulation rate (Knight and Glister, 2003; Knight and Glister, 2006). In growing ovarian follicles *GDF9* growth factor is secreted and plays role in growth and differentiation of early ovarian follicles (McPherron and Lee, 1993). *GDF9* homozygous mutant animals are found to be anovulatory whereas animals heterozygous for *GDF9* have higher than normal ovulation rate (Chu et al., 2005). This shows that *GDF9* is essential for normal folliculogenesis in sheep. So, *GDF9* was an obvious candidate gene with a major effect on litter size in sheep (Davis, 2005), *GDF9* may be a potential major gene on litter size in goats. However, the literatures concerning *GDF9* gene and goat reproduction are relatively rare.

The population of goat in the world was approximately 861.9 million (FAOSTAT, 2008) and India holding the second position with 125.7 millions goat. In India, mainly the landless farmers and small size holders maintain goats, which provide a dependable source of income to most of the rural population who are below the poverty line. Improvement of reproductive traits in livestock species has become of increasing interest, where small increases in litter size can equal large gains in profit. Genetic improvement of reproductive traits has traditionally been restricted to use of quantitative genetic methods but gain has been limited when using these methods. Provided that the major genes associated with reproduction are identified, they can be utilized in breeding through marker-assisted selection (MAS). Reproductive traits are often suggested as prime targets for MAS for their low heritability and the fact that the trait can be measured only in one sex (Ghaffari et al., 2009).

The Assam Hill goat which is known for its high prolificacy has kidding records varying from single to quadruplet. Their average litter size at birth was reported to be 1.41 (Gogoi, 1987). They are found mostly in the hilly regions of Assam and its adjoining areas. Even though the Assam Hill goat is considered as one of the most prolific goat in India, so far no attempts have been made to carry out research work on the fecundity gene, at molecular level, which may be responsible for prolificacy in Assam Hill goat. Therefore, the present investigation was designed to study the polymorphism of GDF9 as important fecundity gene in Assam hill goats.

MATERIALS AND METHODS

Blood collection and DNA extraction

Total of 92 samples Assam Hill goats were collected based on their history of litter size. Goats were obtained from different parts of state Assam, India. Out of this, 50 samples were twins, 10 singlet, 20 triplet and 12 quadruplets. 5 (five) ml of blood sample was collected aseptically from jugular vein of each goat using 0.5 ml of EDTA (ethylene diamine tetra acetate, 0.5 M, pH = 8) as an anticoagulant. The samples were brought to the laboratory in double walled ice-boxes containing icepacks and stored at -20°C until the genomic DNA was extracted. Genomic DNA of goat was extracted by phenol-chloroform extraction procedure (Sambrook and Russel, 2001).

Polymerase chain reaction (PCR) amplification and gel electrophoresis

A 294 bp region of GDF9 gene was amplified by using a set of forward (5′-GTTGGAATCTGAGGCTGAG-3′) and reverse (5′-ATCTGCTCCTACACACCTG-3′) primers (Table 1). PCR reaction was performed in thermocycler (Applied Biosystem, USA) in 25 µl reaction volume containing 40 ng of each primers, 200 µM dNTP mix, 1.5 nM $MgCl_2$, 2.5 mM of 10X buffer, 100 ng DNA template and 1U Taq DNA polymerase (MBI, Fermentas). The PCR cycling parameters were optimized as follows: Initial denaturation at 94°C for 5 min, followed by 94°C for 30 s, 46°C for 30 s, 72°C for 30 s for 35 cycles and final extension at 72°C for 5 min. The PCRproducts were separated by horizontal submarine agarose gel (2%, free from DNAse and RNAse) electrophoresis in 0.5X TBE buffer at 110

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Abbreviations: *GDF9*; Growth differentiation factor 9; **MAS,** marker-assisted selection; **PCR,** polymerase chain reaction; **RFLP,** restriction fragment length polymorphism; **SSCP,** single strand conformation polymorphism.

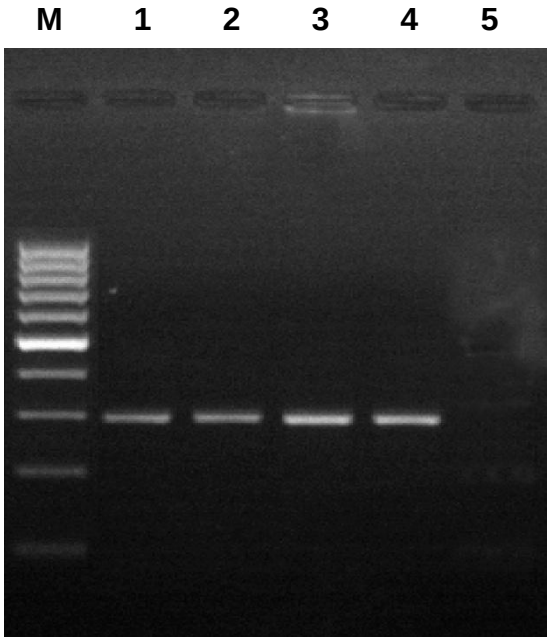

Figure 1. PCR amplicon of *GDF9* gene (294 bp). L1-L4, PCR amplicons of *GDF9* gene of Assam Hill goat; L-5, negative control; M, marker 100 bp.

V and visualised using a gel documentation system (Gel Logic 100, KODAK).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis

PCR products of *GDF*9 gene were used for restriction enzyme digestion by *Dde*I, *Hha*I and *Alu*I at 37°C for 3 h. The restriction enzyme digested products were resolved in 2.5% (w/v) agarose gel in 0.5X TBE buffer. 10 µl of RE digested product along with 5 µ of 6 x gel loading dye, after mixing was loaded in well. A 50 and 100 bp marker ladder was also run alongside the samples to ascertain the size of the amplified products. Electrophoresis was carried out at 110 V for 1.15 h. The digested products were visualized under UV light on a trans-illuminator and the gels were recorded in a gel documentation system.

Sequencing and phylogenetic analysis

PCR amplicon of *GDF9* gene were sequenced at South Campus, Department of Biochemistry, University of Delhi by automated DNA sequencer (ABI Genetic Analyser) following Sanger's dideoxy chain termination method (Sanger et al., 1977). The sequences were analysed by using Clustal W method of DNASTAR Software (Lasergene, USA) and MEGA 5 Software to generate sequence alignment reports, sequence distance, residue substitution and phylogenetic analysis.

RESULTS

Concentration of extracted DNA and purity

After quantification of each DNA sample, a uniform final concentration of 100 ng/µl was prepared by dilution of the entire sample in 0.5M Tris EDTA (TE) buffer.

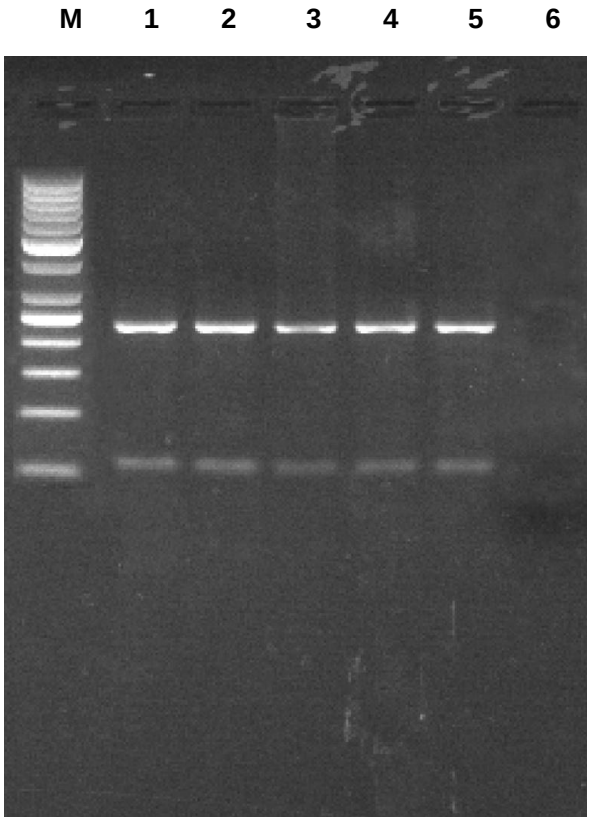

Figure 2. PCR-RFLP of *GDF9* gene using *Dde*I (242 and 52 bp). L1- L5, digested products (252 and 52 bp); M, Marker 50 bp.

Restriction fragment length polymorphism analysis

In order to determine the polymorphism, if any in amplified fragment (Figure 1) of *GDF9* gene, restriction enzyme *Dde*I, *Hha*I and *Alu*I having recognition site of (C↓TNAG), (C↓CGG) and (AG↓CT) were used to digest 294 bp fragment. In the present study, a total of 92 Assam hill goats were genotyped with PCR-RFLP approach. All 92 Assam hill goats were screened for *Dde*I, *Hha*I and *Alu*I enzyme digestion followed by agarose gel electrophoresis revealed single type of restriction pattern consisting two fragments. *Dde*I digestion produced two fragments approximately 242 and 52 bp (Figure 2). Similarly, *Hha*I gave two fragments of 140 and 154 bp (Figure 3) and *Alu*I cut the amplicon of 294 bp into two fragments measuring approximately 134 and 160 bp (Figure 4), respectively. The genotype was deduced to be homozygous normal wild type in all the animals and suggest that in spite of totally different breeds of European and Indian origin, no new site was created for the enzymes in *GDF9* gene.

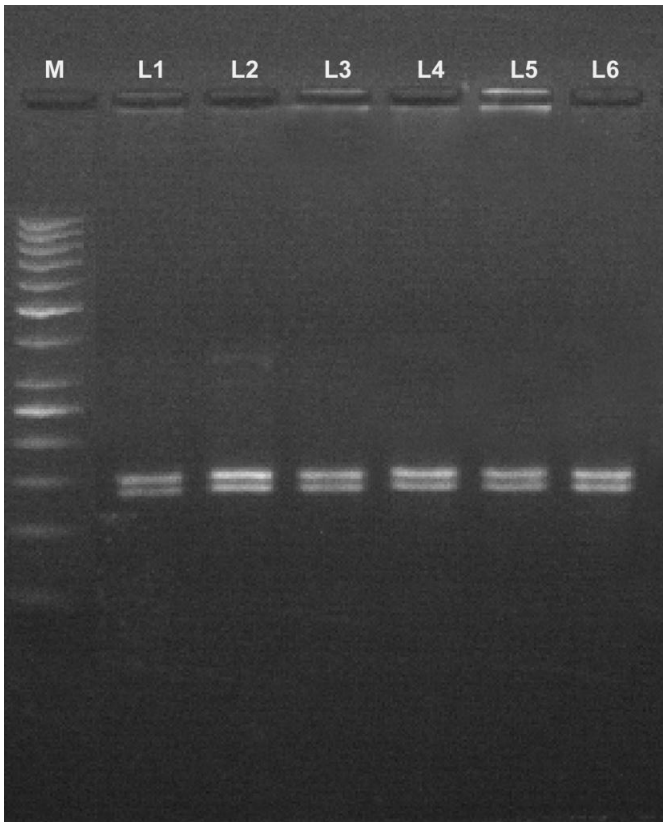

Figure 3. PCR-RFLP of *GDF9* gene using *Hha*I (154 and 140 bp). L1-L6, Digested products (154 and 140 bp); M, marker 50 bp.

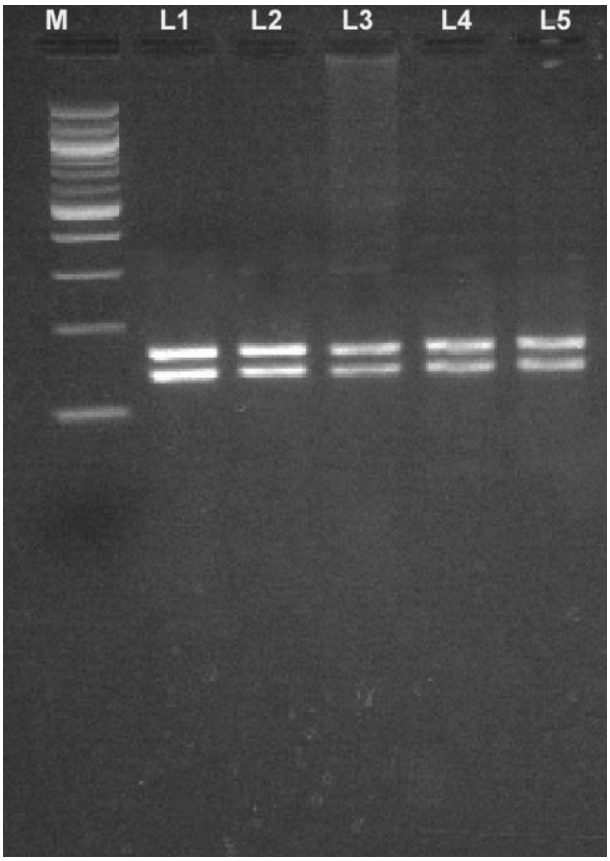

Figure 4. PCR-RFLP of *GDF9* gene using *Alu*I (160 and 134 bp). L1-L5, Digested product of (160 and 134 bp); M, marker 100 bp.

Nucleotide sequence comparison

The sequence of same region was analyzed. The nucleotide sequences (NCBI accession no. JX483872) of our experiment were aligned and compared using DNAstar software (USA). Eight published sequences of *GDF9* gene of other breeds of goat viz. Barbari (NCBI accession no. HM462265), Black Bengal (NCBI accession no. FJ665810), Ganjam (NCBI accession no.JN601041), Jaminapari (NCBI accession no.HM462268), Lezhi Black (NCBI accession no.JF824149), Osmanabadi (NCBI accession no.HM462267), Sirohi (NCBI accession no.JN680860) and Tibetan (NCBI accession no. JN100108) *GDF9* genes were obtained. All the nucleotides of *GDF9* genes obtained in the present study showed 97-100% similarity with the other goats. Similarity study of *GDF9* gene of Assam Hill goat showed that it has similarity of 97.0, 97.4, 97.4, 97.4, 95.3, 97.4, 97.0 and 97.0% with Barbari, Black Bengal, Ganjam, Jaminapari, Lezhi Black, Osmanabadi, Sirohi and Tibetan goats, respectively (Figure 5).

The partial sequences of *GDF9* gene of Assam Hill goat were aligned to the position of 260 to 495 bp of the complete *GDF9* gene sequence of Sirohi goat (NCBI accession no. JN680860). At position 495 (C > A) and at 387 (G > A), nucleotide substitution were found in all the sequences of Assam Hill goat, which was found to be unique in comparison to the other sequences. In one sequence of Assam Hill goat, an insertion of nucleotide 'A' was found at position 268, which might be an individual variation. The Assam Hill goat having insertion of nucleotide "A" at position 268 showed remarkable change in amino acid sequence. None of the ten SNPs identified so far in sheep *GDF9* gene including four mutations (FecGH, FecGE, FecTT and G1) associated with fecundity (Table 2) could be identified in Assam Hill goats.

Phylogenetic analysis

The nucleotide sequenced of GDF9 gene of Assam hill goat used for construction of phylogenetic tree along with published sequences of this gene. Phylogenetic analysis revealed that the sequences Assam Hill goat belongs to a commons cluster which differ with that of the other goat

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	
1		97.9	97.0	97.0	98.0	99.8	99.9	99.8	99.6	99.9	97.0	1
2	2.2		97.4	96.6	99.9	98.4	99.8	98.3	97.5	99.8	97.4	2
3	2.6	2.2		99.6	97.4	97.4	95.3	97.4	97.0	95.3	100.0	3
4	2.6	2.2	0.0		96.6	96.6	94.5	96.6	96.2	94.5	99.6	4
5	2.1	0.1	2.2	2.2		98.5	99.9	98.4	97.6	99.9	97.4	5
6	0.1	2.1	2.2	2.2	2.0		99.9	100.0	99.8	99.9	97.4	6
7	0.1	0.2	0.0	0.0	0.1	0.1		99.9	99.9	99.9	95.3	7
8	0.1	2.1	2.2	2.2	2.0	0.0	0.1		99.8	99.9	97.4	8
9	0.1	2.2	2.6	2.6	2.0	0.1	0.1	0.1		99.9	97.0	9
10	0.1	0.2	0.0	0.0	0.1	0.1	0.1	0.1	0.1		95.3	10
11	2.6	2.2	0.0	0.0	2.2	2.2	0.0	2.2	2.6	0.0		11
	1	2	3	4	5	6	7	8	9	10	11	

Divergence

1 gdf9 Barbari Goat
2 gdf9 Black Bengal
3 gdf9 g1 Assam Hill Goat
4 gdf9 g2 Assam Hill Goat
5 gdf9 Ganjam Goat
6 gdf9 Jamnapari Goat
7 gdf9 Lezhi Black Goat
8 gdf9 Osmanabadi goat
9 gdf9 Sirohi Goat
10 gdf9 Tibetan Goat
11 gdf9 G3 Assam Hill Goat

Figure 5. Percent identity and divergence of *GDF9* gene.

Table 2. Major mutations of *GDF9* gene identified in sheep.

	Base change	Coding base (bp)	Coding residue (amino acid)	Mature peptide residue (amino acid)	Amino acid change	Reference
G1	G→A	260	87	-	Arg→His	Hanrahan et al. (2004)
G2	C→T	471	157	-	Unchanged Val	-
G3	G→A	477	159	-	Unchanged Leu	-
G4	G→A	721	241	-	Glu→Lys	-
G5	A→G	978	326	8	Unchanged Glu	-
G6	G→A	994	332	14	Val→Ile	-
G7	G→A	1111	371	53	Val→Met	-
G8	C→T	1184	395	77	Ser→Phe	-
FecGSI	T→G	1034	345	27	Phe→Cys	Melo et al. (2008)
FecTT	A→C	1279	427	109	Ser→Arg	Nicol et al. (2009)
A152G	A→G	152	51	-	Asn→Asp	Li et al. (2003)
T692C	T→C	692	231	-	Leu→Thr	Gao (2007)

breeds when clustering was done at 1.2 x 100 nucleotide substitution. The close proximity of location of GDF9 gene in the same clade of tree indicated the descendent of the genes in those groups from a common ancestor (Figure 6).

DISCUSSION

Nucleotide substitution from G to A at position 387 show that the predicted change in amino acid is observed to be glycine (G) to aspartic acid (D) at position 129 in GDF9 protein. Li et al. (2003) identified one single nucleotide mutation (A152G) of GDF9 gene in Hu, Dorset and Suffolk sheep by PCR-single strand conformation polymorphism (SSCP), which resulted in an amino acid change Asn51Asp. Hanrahan et al. (2004) reported eight DNA variants in *GDF9* of Cambridge and Belclare sheep including G1 to G8. Out of these eight polymorphisms, three nucleotide changes did not alter amino acids (G2, G3 and G5). Four G >A mutations of the eight SNPs resulted in amino acid changes (G1, G4, G6 and G7)

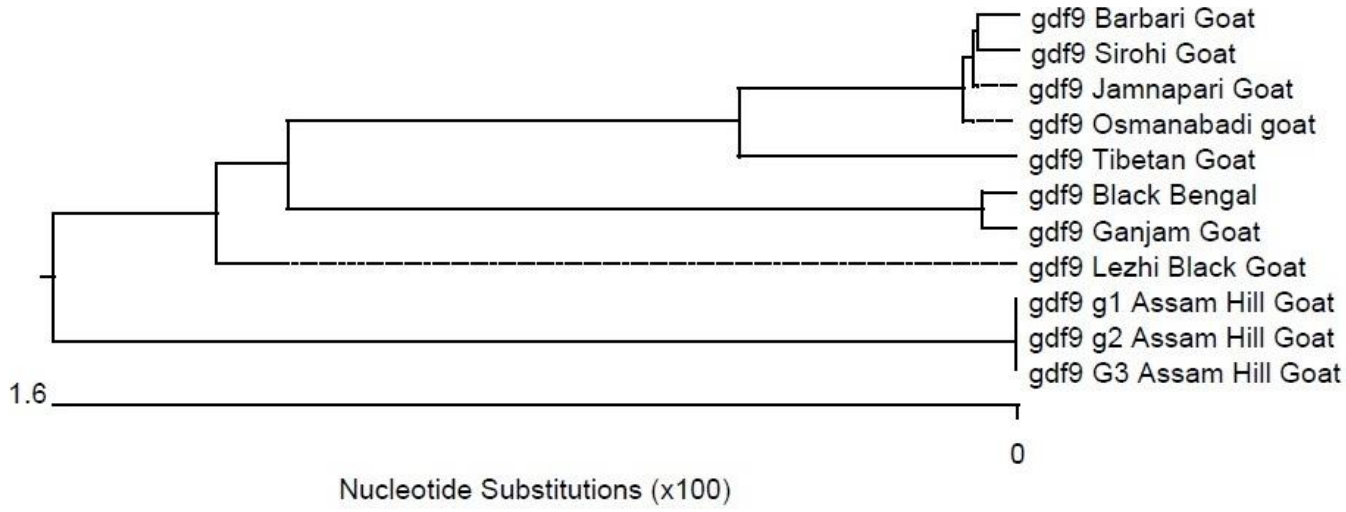

Figure 6. Phylogenetic tree of *GDF9* gene.

which occurred at position before the furin processing site or unprocessed protein and were unlikely to affect the mature active coding region. However, G8 variant also known as FecGH caused serine to phenylalanine at residue 395 which replaced an uncharged polar amino acid with a non polar one at residue 77 of the mature coding region and may change the function GDF9 in sheep. Recently, Melo et al. (2008) and Silva et al. (2010) detected FecGE mutation (c.1024T>G resulting in p.F345C) in *GDF9* gene of Brazilian Santa Ines sheep. Another new mutation (c.1279A>C, named as FecTT) was found in *GDF9* gene of Icelandic Thoka sheep resulting in a non-conservative p.S109R in the C-terminus of the mature *GDF9* protein (Nicol et al., 2009). Three mutations (c.423G>A, c.959A>C [p.Q320P] and c.1189G>A [p.V397I]) in exon 2 of *GDF9* gene had been detected extensively in several goat breeds, in which mutation c.423G>A was detected in Jining Grey, Liaoning Cashmere and Boer goats (Wu et al., 2006; Feng et al., 2010), Wendeng Dairy and Beijing native goats (Wu et al., 2006) and Guizhou White goats (Feng et al., 2010); mutation c.959A>C was found in Yangtse River Delta White and Huanghuai goats (Zhang et al., 2008), Jining Grey, Liaoning Cashmere and Guizhou White goats (Feng et al., 2010) and Boer goats (Zhang et al., 2008; Feng et al., 2010); mutation c.1189G>A was identified in Jining Grey, Liaoning Cashmere and Boer goats (Wu et al., 2006; Feng et al., 2010), Guizhou White goats (Du et al., 2008; Feng et al., 2010), Wendeng Dairy and Beijing native goats (Wu et al., 2006; Feng et al., 2010).

Conclusion

In the present study, the results showed monomorphic banding pattern in all the studied samples of Assam Hill goat with respect to *GDF9* gene. Regarding the records of high prolificacy in Assam Hill goat, it is concluded that probably the genetic factor controlling twinning and triplet is not related to the mutation which is reported in the *GDF9* gene. It may be concluded that probably the litter size in Assam Hill is either not affected by major genes or it is possible that other SNP in the *GDF9* gene or some other major genes may be controlling the prolificacy in Assam Hill goat.

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Full Length Research Paper

Isolation and characterization of partial mitochondrial *CO1* gene from harpacticoid copepod, *Leptocaris canariensis* (Lang, 1965)

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Copepods are gaining attention as superior viable live feed for larviculture and as ecological bioindicators. One of the possible candidates from Malaysia is *Leptocaris canariensis* (Copepoda: Harpacticoida). However, little is known about the molecular aspect of this species. In this study, DNA of individual *L. canariensis* was extracted and the partial mitochondrial *CO1* gene was successfully amplified using universal primers LCO-1490 and HCO-2198. A 582 bp partial mitochondrial *CO1* gene sequence was obtained. Analysis of partial CO1 sequences of *L. canariensis* revealed 100% similarity among all the individual copepods, verifying the purity of samples and the consistency of the optimized extraction and amplification protocols done in this study. BLAST analysis confirmed that the obtained sequences were from CO1 region and of copepod origin (with E-value < e^{-10}). Phylogenetic analysis of *L. canariensis* along with selected outgroups from different taxa level further supports the purity of *L. canariensis* maintained and validates the taxonomy of *L. canariensis* up to the subclass level: Copepoda. This study serves as the first documentation of molecular studies done on harpacticoids from the genus *Leptocaris*. The availability of *L. canariensis* partial CO1 sequence as reference will spearheads many more research in various fields in the near future.

Key words: Harpacticoida, *Leptocaris canariensis*, *CO1* gene, molecular identification.

INTRODUCTION

Copepods are the second largest meiofaunal group in marine sediment environment, after nematodes (Barnes, 1982) and they serve as food for juvenile fish in the marine meiobenthic food web and aquatic pollutant transporters across the food chains (Goetze, 2003; Raisuddin et al., 2007: Lundström et al., 2010; Lauritano et al., 2012). Being an order of copepods, harpacticoids can be found throughout the world in the marine environment and in fresh water (Boxshall and Defaye, 2008). Harpacticoid copepods are gaining attentions as

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Abbreviations: CO1, Cytochrome c oxidase subunit 1; **PCR,** polymerase chain reaction; **BLAST,** basic local alignment search tool.

possible substitutes for calanoid copepods due to their high stocking densities (Støttrup et al., 1986; Cutts, 2003; Støttrup, 2003) and the ability to convert shorter chain n-3 polyunsaturated fatty acids to the essential fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Nanton and Castell, 1998; Cutts, 2003). It was also noted that the nauplii of harpacticoid copepod can be more easily and completely digested compared to rotifers or Artemia (Pederson, 1984; Schipp, 2006).

High numbers of species *Leptocaris* (Harpacticoida) were recorded in mangrove area of west peninsular Malaysia. They were exclusively found on mangrove leave litters (Somerfield et al., 1998). However, there was no known literature on the molecular aspects of harpacticoids from the genus Leptocaris, including those found in Malaysia.

Compared to molecular identification method, morphological identification has some notable shortcomings. Absolute morphological identification of copepod can only be done once they reached late copepodite and adult stage because they showed only limited differences in their morphology during larval stage (Frost, 1971). Apart from that, morphological identification is time consuming, ambiguous and is more prone to misidentification of rare species (Bucklin et al., 2003; Jagadeesan et al., 2009). Molecular analysis has been proven to be important and useful in various fields of studies, especially in the understanding of deep phylogenetic relationships (Blair and Hedges, 2005; Regier et al., 2005), examining intra- and inter-specific relationships and population structure within and between species (Avise et al., 1987; Zhang and Hewitt, 2003; Dippenaar et al., 2010), identification of unknown or immature specimens based on established reference molecular data (Olson, 1991; Bartlett et al., 1992), and in delimiting cryptic species (Goetze, 2003; Hendrixson and Bond, 2005; Thum and Derry, 2008).

Cytochrome c oxidase subunit 1 (*CO1*) gene is considered as one of the widely used markers in the studies of population genetics and evolution (Shao et al., 2007) because it is among the most conservative protein-coding genes found in the mitochondrial genomes of animals (Brown, 1985). The phylogenetic relationships of 34 calanoid species belonged to 10 genera and 2 families were successfully determined by Bucklin et al. (2003) with the aid of mitochondrial *CO1* gene of size 693 bp. *CO1* gene has also been proven to be successful in species recognition (Hebert et al., 2003; Waugh, 2007) and specifically efficient in copepods (Bucklin et al., 1999; Hill et al., 2001; Øines and Heuch, 2005). Apart from that, the mitochondrial *CO1* gene variation within a species is far less than the variation that exists between species, making *CO1* gene a good diagnostic molecular marker (Bucklin et al., 1998, 1999).

The aims of this study were (i) to isolate the partial mitochondrial *CO1* gene of *Leptocaris canariensis* (Lang, 1965), and (ii) to characterize the partial CO1 gene of *L. canariensis*. Molecular characteristics of *L. canariensis* were generated through this study. This study serves as the first documentation and characterization of partial mitochondrial *CO1* gene from *L. canariensis* in Malaysia. The result generated from this study can aid in future identification, differentiation and phylogenetic studies of *L. canariensis*.

MATERIALS AND METHODS

Sample collection

Pure cultures of harpacticoid copepods *L. canariensis* were supplied by researchers of Institute of Tropical Aquaculture (AKUATROP), Universiti Malaysia Terengganu (UMT). The pure cultures maintained were initially collected from Merchang (5°1′N; 103°17′E) Terengganu, Malaysia.

DNA extraction, PCR amplification, and sequencing

Individual copepods were extracted and minced in 10 µl of sterile distilled water using fine needle viewed under dissecting microscope. The 10 µl mixture was transferred to 0.2 ml PCR tube. They were then incubated overnight at 4°C after addition of 2.5 µl 10X PCR buffer A (Vivantis Tech., MY) and 6.98 µl of sterile distilled water. The remaining PCR ingredients were added for PCR amplification on the following day. Universal primers LCO-1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO-2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994) were used to amplify and sequence a 710 bp fragment of partial mitochondrial *CO1* gene from *L. canariensis*. Each 25 µl of PCR reaction contained 16.98 µl of sterile distilled water (with minced copepod), 2.5 µl of 10X Buffer A (Vivantis Tech., MY), 1 µl of $MgCl_2$ (50 mM), 1 µl of dNTPs (10 mM), 1.56 µl of each primers (40 µM) and 0.4 µl of *Taq* polymerase (Vivantis Tech., MY). PCR temperature profile used: initial denaturation at 94°C for 900 s; 40 cycles of denaturation at 94°C for 45 s, annealing at 52°C for 45 s, extension at 72°C for 60 s; final extension at 72°C for 600 s (modified from Vestheim et al., 2005).

The DNA of 30 individual harpacticoid *L. canariensis* were extracted and amplified via this method. Obtained PCR products were visualized on an ethidium-bromide (EtBr) stained 1.5% agarose gel under Ultra-Violet light (UV), and purified using Vivantis GF-1 PCR clean-up kit (Vivantis Tech., MY) before sent for sequencing (First BASE Lab. Sdn. Bhd.).

Data processing and sequence analyses

The obtained sequencing results were viewed and edited using Chromas Ver. 2.33 (Technelysium Pty Ltd) to check for their quality, trimmed off early and end signal losses, ambiguous and unusable base pairs before further analyses. The CO1 gene sequences of 30 samples were aligned with National Center of Biotechnology Information (NCBI) BlastN database by using BLAST (Basic Local Alignment Search Tool) algorithm in the internet for comparison and identification (Altschul et al., 1997). The nucleotide sequences were validated by comparing the amino acid (translated protein) sequences with consensus protein sequences for the same mitochondrial *CO1* gene region.

Phylogenetic analysis using multiple alignment was carried out

Table 1. Pairwise nucleotide distances (Kimura 2-parameter) for partial CO1 gene sequences between *L. canariensis* and outgroups. Two *L. canariensis* CO1 gene sequences (w85 & w71) were chosen to represent all the 30 sequences as they produced identical results. Pairwise nucleotide distances were shown in lower left column while standard error estimates were shown on upper right column.

Parameter	1	2	3	4	5	6	7
Leptocaris canariensis (w85)	-	0.000	0.032	0.031	0.035	0.038	0.098
Leptocaris canariensis (w71)	0.000	-	0.032	0.031	0.035	0.038	0.098
Cletopsyllidae sp.	0.362	0.362	-	0.028	0.030	0.035	0.165
Cletocamptus deitersi	0.369	0.369	0.303	-	0.031	0.034	0.141
Coullana sp.	0.422	0.422	0.312	0.331	-	0.032	0.153
Tigriopus californicus	0.450	0.450	0.407	0.391	0.390	-	0.177
Calanus sinicus	1.099	1.099	1.363	1.282	1.327	1.386	-

too using phylogenetic analysis software ClustalX 2.0.12 (Larkin et al., 2007). Uncorrected, pairwise sequence divergences among the obtained mitochondrial *CO1* gene sequence and outgroups were calculated using molecular evolutionary genetics analysis (MEGA), Ver.4.0.2 (Tamura et al., 2007). Similarly, phylogenetic analyses using Neighbour-Joining (Saitou et al., 1987) search with Kimura 2-parameter as model was conducted using MEGA. The tree was bootstrapped using 1000 subreplicates. Outgroups *Cletocamptus deitersi* (AF315012.1), *Cletopsyllidae* sp. (AY327386.1), *Coullana* sp. (AF315015.1), *Tigriopus californicus* (DQ913891.2) and *Calanus sinicus* (EU603284.1) were selected from GenBank database.

RESULTS

Nucleotide identification

BLAST analysis was conducted on all sequencing data of *L. canariensis* by comparing the partial mitochondrial *CO1* gene sequences of *L. canariensis* with the online database of GenBank. All of the BLAST hits showed significant similarity (E-value < e^{-10}) (Chini et al., 2006) with the 30 individual *L. canariensis* partial *CO1* gene sequences. All of the hits retrieved from GenBank database were nucleotide sequences of partial mitochondrial *CO1* gene regardless of species, hence verifying the CO1 origin of *L. canariensis* samples.

The PCR products of 30 *L. canariensis* individuals showed 77% similarity with the partial mitochondrial CO1 gene region of TWO isolates of calanoid copepods, *Boeckella brasiliensis* (GenBank Accession Number: DQ356546.1, DQ356545.1). Five additional hits retrieved with a slightly higher (78-79%) maximum identity percentage values were all from the order Diptera. Nevertheless, the percen-tage of query coverage was highest (87-90%) in calanoid *B. brasiliensis* and showed a considerable difference compared to the other five retrieved hits (78-81%).

Sequence similarity

A perfect matching sequence of 582 bp partial mitochondrial CO1 gene sequence was recovered when multiple alignment analysis was conducted on all obtained partial mitochondrial CO1 gene sequences of *L. canariensis*. The mitochondrial CO1 sequence was submitted to GenBank, NCBI with Accession Number JF707331.

Consistent results were obtained in all of the obtained partial CO1 sequence of *L. canariensis* when compared with selected outgroups in pairwise nucleotide distances analysis (Table 1). Identical results of 0.000 (100% similarity) were seen in the comparisons between individual *L. canariensis* partial mitochondrial CO1 gene sequences (*L. canariensis* w85 & w71) (Table 1).

A range in between 0.30-0.45 were observed for the pairwise nucleotide distances (Kimura 2-parameter) comparison of *L. canariensis* CO1 gene sequences with the CO1 gene sequences of selected harpacticoid copepods outgroups (*Cletopsyllidae* sp., *C. deitersi, Coullana* sp. and *T. californicus*). On the other hand, a huge pairwise nucleotide distances of 1.10 - 1.39 were observed between copepods of the order Harpacticoida (including *L. canariensis*) and the order Calanoida (*C. sinicus*).

Phylogenetic analysis

The partial mitochondrial CO1 sequences of *L. canariensis* and selected outgroups were used to create a gene tree using Neighbour-Joining method (Figure 1). The tree branched out into two main clusters according to different orders as expected, with *C. sinicus* (Order: Calanoida) on one cluster while the rest of the copepods from the order Harpacticoida on another main clusters. All the harpacticoid copepod outgroups were resolved with a high bootstrap value of 99%, thus differentiating *L. canariensis* as well.

DISCUSSION

The confirmation of species identity using molecular

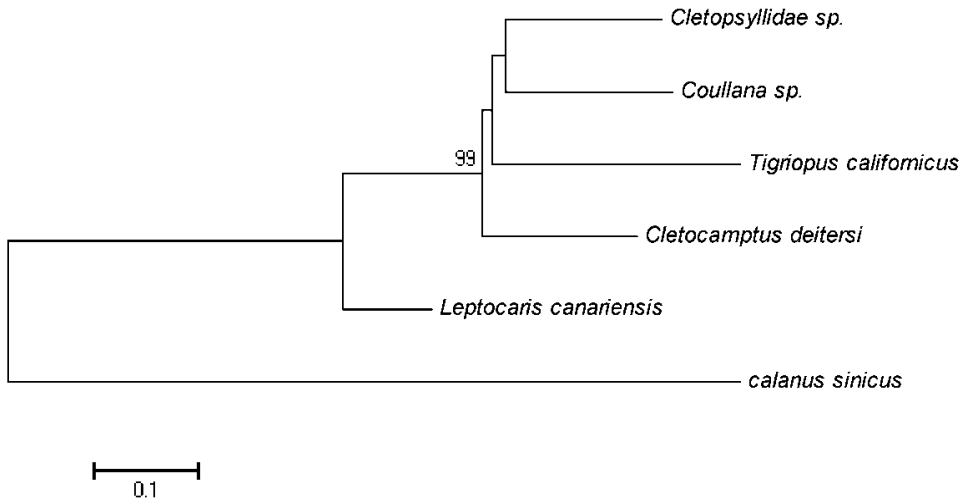

Figure 1. Phenogram of partial mitochondrial CO1 gene region of *L. canariensis* and selected outgroups. Unrooted mitochondrial CO1 gene tree reconstruction was by Neighbour-Joining (Saitou and Nei, 1987) using Kimura 2-parameter; tree was bootstrapped 1000x. Bootstrap value is given at the branch point.

method is crucial in ensuring that the copepod cultures maintained were of single species, especially in organisms as minute and ambiguous as copepods. The recovered 582 bp partial mitochondrial *CO1* gene sequence in this study was in conformation with Laakmaan et al. (2012), where partial *CO1* gene sequences of 525-658 bp were recovered using the same universal primers. The PCR products of *L. canariensis* amplified in this study were confirmed to be the gene sequence of partial CO1 region as all of the hits recovered from BLAST search were *CO1* gene sequences too as expected, with reliable E-value. This confirmation also reflects the ability and effectiveness of universal primers LCO-1490 and HCO-2198 described by Folmer et al. (1994) to target partial CO1 region in most invertebrates, including copepods. The high similarity (77%) observed between a species of calanoid copepod (*B. brasiliensis*) and the harpacticoid studied in this research (*L. canariensis*) further verifies that the partial CO1 gene sequences obtained in this study was of the subclass Copepoda. The appearance of organisms from other subphylum (Hexapoda) appeared in BLAST search was expected as universal primers LCO-1490 and HCO-2198 were used. The closest match was 79% similarity to a *Diptera* sp. CO1 sequence (GenBank Accession Number HM420105.1). Bucklin et al. (1999) also experienced similar situations whereby 88% similarity (closest match) to a *Murex troscheli* (sea snail) CO1 sequence was found when they compare the obtained *CO1* gene sequence of *Calanus finmarchicus* with *CO1* gene sequences in GenBank database.

Absolute (100%) similarities (pairwise nucleotide value = 0.000) were observed among all the 30 individual *L. canariensis* partial *CO1* gene sequences while huge differences of pairwise nucleotide distances values were observed when compared to other outgroups (Table 1). The absence of pairwise nucleotide distance found in all 30 *L. canariensis* samples indicated that all the samples were of the same species. This is expected as Bucklin et al. (1999) reported that the *CO1* gene region differed between species by approximately 30% of nucleotides, but exhibited little or no variation within species. Eberl et al. (2007) also showed that based on the uncorrected divergence (*p*-distances), CO1 sequences of harpacticoid *Macrosetella gracilis* sampled at Atlantic and Pacific have a genetic distance of less than 2% (Kimura 2-parameter distances). The degree of relatedness between *L. canariensis* and the other outgroups is reflected in this study and indirectly supports the taxonomy of *L. canariensis*. *L. canariensis* is expected to be more similar (smaller genetic distances) to other harpacticoid copepods compared to calanoid copepod, as shown in this study (Table 1).

Multiple alignment analysis of all the obtained partial CO1 sequences of *L. canariensis* on the other hand enabled the recovery of a matching sequence with the length of 582 bp partial *CO1* gene sequence. This recovery was expected as Folmer et al. (1994) reported in their study that universal primers LCO-1490 and HCO-2198 were able to amplify partial mitochondrial *CO1* gene sequence of 710 bp and readable sequences of approximately 651 bp were obtainable in more than 80 species ranging from 11 different phyla. This perfectly matched 582 bp CO1 sequence implied that all the *L.*

canariensis samples tested were of pure culture and no divergence or contamination of other morphologically similar species exists in the cultured stock population. This obtained *CO1* gene sequence can serve as a reference partial *CO1* gene sequences of *L. canariensis* for any future studies such as identification, population studies, intraspecific and interspecific discrimination of *L. canariensis*.

Phylogenetic analysis on the partial mitochondrial *CO1* gene sequences of *L. canariensis* and selected outgroups using Neighbour-Joining method confirmed the taxonomic hierarchy of *L. canariensis* up to order level (Copepoda) (Figure 1). Based on this phenogram, it is deducible that all the samples were equally identical in terms of genetic distance and as harpacticoids, *L. canariensis* is more closely related to other harpacticoid copepods compared to the calanoid copepod (*C. sinicus*). This confirmation is important because studies done on harpacticoid *Tigriopus californicus* (Burton and Lee, 1994; Ganz and Burton, 1995) found that mitochondrial DNA (especially *CO1* gene) revealed extreme genetic divergence even over short geographical distances. Studies have also shown that some marine invertebrates (including copepods) will undergo cryptic speciation, diverging at molecular level but remains morphologically similar (Knowlton, 1993; Lee, 2000; Goetze, 2003).

The present study showed the ability of partial *CO1* gene in differentiating between species (Figure 1). However, the 0.000 pairwise divergence between same species (*L. canariensis*) indicates that this biomarker is not suitable to be used in resolving relationships within similar species. The limited usefulness of mitochondrial *CO1* gene region, with a resolving power for some groups at genus level and above, may be due to the rapid evolving rate within this region (Miyata et al., 1982; Machida et al., 2006). The successful confirmation of *L. canariensis* up to the order level (Harpacticoida) was consistent with the conclusion made by Costa et al. (2007), stating that the mitochondrial CO1 region has a 95% success rate in classifying species to the correct order.

Conclusion

The accuracy of using molecular methods to identify and validate harpacticoid copepods have been shown in this study. The purity of the *L. canariensis* population is confirmed with 100% similarity among the partial mitochondrial *CO1* gene sequences obtained. The ability of using mitochondrial CO1 gene as DNA barcoding marker was proven when amplification of *CO1* gene was feasible in all of the samples tested.

However, it is suggested to include another gene from nuclear genome to make the data more robust. Apart from that, partial mitochondrial *CO1* gene may serve as a good biomarker for distinguishing between species, but were poor in resolving differences within species, especially in *L. canariensis*. The presented information here will spear-head more future research on creating a copepod DNA bank and ease their monitoring as possible ecological bioindicators and as viable live feed candidates.

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Marker assisted characterization of chickpea genotypes for wilt resistance

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Marker assisted characterization of six chickpea genotypes differing for *Fusarium* wilt reaction was carried out using seven molecular markers reported by earlier workers linked to disease resistant/susceptibility. In the present study, four different markers (namely, CS-27, UBC-170, CS-27A and UBC-825) linked to susceptibility and three microsatellite based markers (TA-59, TA-96 and TR-19) linked to resistance allele were validated. It was observed that two Random Amplified Polymorphic DNA (RAPD) markers, CS-27 and UBC-170 and one sequenced characterized amplified region (SCAR) CS-27A$_{700}$ gave amplification of 700, 550 and 700 bp, respectively in susceptible genotype only as reported by earlier worker. The inter simple sequence repeat (ISSR) marker UBC-825 produced amplification of 1200 bp in susceptible genotypes (JG-62 and GG 4) and intermediate genotype (Chaffa). Three sequence tagged microsatellites site (STMS) primers (TA-59, TA-96 and TR-19) gave specific allele in wilt resistant genotypes. The PCR amplification of TA-59 primer generated two alleles, out of which the allele of 258 bp was present only in resistance genotypes. The alleles of 265 bp amplified by primer TA-96 was present only in resistance genotypes and absent in other genotypes. The marker TR-19 amplified allele of 227 bp in resistant genotypes. Further, the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) analysis of seed storage protein showed a difference in protein profile among studied genotypes but none of polypeptide fragment was specific to wilt resistance or susceptibility. In present study, the reported markers linked to susceptibility and resistance proved their effectiveness and further can be exploited for maker assisted selection (MAS) of wilt resistance breeding in chickpea.

Key words: Chickpea, *Fusarium* wilt, molecular markers, resistance, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE).

INTRODUCTION

Chickpea (*Cicer arietinum* L.; 2n = 2x = 16; genome size of 750 Mbp) is an important legume crop in most of the developing countries in the world and ranks third among food legumes in production (FAO, 2012). Chickpea is most important pulse crop of India and its adjoining countries account for 90% of the total world production (Gupta et al., 2011). However, the average annual world chickpea yield (0.78 tons/ha) is considered comparatively lower than its potential yield (Sudupak et al., 2002). One major reason for the low productivity of cultivated chickpea is its narrow genetic base and its sexual incompatibility with other wild species of *Cicer* in natural inter specific

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crosses (Abbo et al., 2003). Furthermore, various biotic (*Fusarium* wilt, *Aschochyta* blight, nematodes and pests) and abiotic stresses (drought and cold) severely reduce the yield (Croser et al., 2003). Vascular wilt caused by the deuteromycetes fungal pathogen *Fusarium oxysporum* f. sp. *Ciceri* is one of the major constraints in realization of the full yield potential (4 tons/ha) of chickpea. This disease causes huge yield losses (10 to 90%) annually (Singh and Reddy, 1991). Therefore, many chickpea breeding programmes are focused on improving the genetic potential, both to increase yield and to provide protection against biotic and abiotic stresses (Rao et al., 2007).

The use of wilt resistant chickpea genotypes, when they are available, is the most effective and eco friendly method of managing the disease (Sabbavarapu et al., 2013). Identifying *Fusarium* wilt race specific resistance genes and transferring them to adapted backgrounds is a major challenge for plant breeders. Direct assays (screening) for these genes may be difficult, particularly when large number of breeding lines is involved. Isozymes and total protein banding pattern have direct/indirect application in plant breeding programmes and host–pathogen interactions. These have been used for characterisation of particular plant genotypes, cultivars, inbred lines, screening variability in plant populations and mapping of chromosomes (Moore and Collins, 1983). Marker assisted selection (MAS) using DNA markers tightly linked to wilt resistance genes can be used to screen a large number of germplasm lines for the presence of these genes without actually subjecting them to the pathogen and to pyramid them into agronomically superior varieties. MAS are an accurate, easy as compared to conventional method, less time consuming and independent to environmental conditions. The genetic studies showed that the resistance to race 4 was monogenic recessive in some lines (Tullu et al., 1998; Sharma and Muehlbauer, 2005); whereas it was digenic recessive in Surutato-77 (Tullu et al., 1999).

Several studies are under taken to decipher the molecular marker closely linked to *foc*-4 resistance. Various markers namely, RAPD, SCAR, ISSR, STMS closely linked to *foc*-4 were reported (Sharma and Muehlbauer, 2007). Further validation of these markers in other genotypes is necessary to prove their efficiency to characterize chickpea genotypes. Thus, the present study was under taken with an objective to validate reported markers with selected chickpea genotypes showing different reaction to wilt disease.

MATERIALS AND METHODS

Plant material

Six chickpea genotypes differing in wilt disease reaction (Rathod and Vakharia, 2011) that is, WR-315 and ICCV-2 (resistant), GG-1 (tolerant), GG-4 (susceptible), JG-62 (highly susceptible) and Chaffa (moderately susceptible) were procured from Castor and Pulses Research station, NAU, Navsari, for molecular characterization against *fusarium* wilt. All genotypes were sown in plastic pots and leaf samples were taken after 20 days of sowing.

Seed storage protein extraction and SDS PAGE

The seed storage protein was extracted according to procedure of Hameed et al. (2009). The procedure of Laemmli (1970) was used for the electrophoresis analysis of protein on vertical SDS PAGE (10%). The standard staining and de-staining procedures were used for visualization of clear protein fragments.

DNA isolation

Total genomic DNA was isolated from the young leaves following the CTAB method described by Rogers and Bendich (1988) with minor modifications. The quality and quantity were estimated by measuring O.D. at 260/280 and 260 nm, respectively in a Nanodrop spectrophotometer. Intactness of genomic DNA was checked on 0.8% agarose gel.

PCR amplification and electrophoresis

PCR amplification was performed following the procedure given by Sethy et al. (2006) with minor modifications. Seven molecular markers reported by earlier worker linked to wilt resistance were synthesized from MWG Biotech, Germany (Table 1). PCR amplifications were performed in 25 µl volumes using a thermal cycler (Bio-Rad, USA). The reaction mixtures contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 60 ng genomic DNA, 25 pmol of each primer, 1.5 mM $MgCl_2$, 2.5 mM of each dNTP and 1.5 units of Taq DNA polymerase (Biogene, USA). The temperature profile consisted of an initial denaturation step of DNA at 94°C for 3 min, followed by 40 cycles: 94°C for 1 min, 55 to 59°C for 1 min, and 72°C for 1 min. Annealing temperatures were optimized individually for each primer. After the final cycle, samples were incubated at 72°C for 10 min to ensure complete extension followed by hold at 4°C. PCR amplified products were mixed with 4 µl of 6x loading dye (0.25% bromophenol blue, 0.25% xylene cynol and 40% sucrose, w/v), electrophoresed on 1.5 or 3.0% agarose gel, stained with ethidium bromide and visualized under UV light.

Data analysis

The amplified products were visualized under UV light and photographed by Bio- Rad gel documentation system. The presence or absence of specific band linked to resistance/susceptible allele reported by earlier worker was recorded. For SDS PAGE analysis, the binary data was prepared using the presence and absence of polypeptide fragment as 1 and 0, respectively. The data was analyzed using numerical taxonomy and multivarient analysis (NTSYS-pc) system version 2.02i by Exeter software (Rohlf, 2004). Jaccard's similarity coefficient was calculated using SIMQUALK programme. A dendrogram was produced using mean of the Unweighted pair group method with arithmetic averages (UPGMA) analysis.

RESULTS AND DISCUSSION

Large number of molecular markers linked to different wilt resistance genes were identified and mapped (Sharma and Muehlbauer, 2007). The available molecular marker information may be used for MAS. In present study, two RAPD, one each for SCAR and ISSR, and three STMS

Table 1. Information regarding molecular markers employed for marker assisted characterisation of six diverse chickpea genotypes with their linkage distance from resistant (R) gene in centi Morgan (cM) and expected fragment size in base pair (bp). The molecular marker reported by earlier worker linked to disease resistance/susceptibility was used.

Source	Primer	Primer sequence	Distance from R gene (cM)	Expected fragment size (bp)
Tullu et al. (1999)	CS-27	AGT GGT CGC G	15.2	700
	UBC-170	ATC TCT CCT G	9.0	550
Mayer et al. (1997)	CS-27A	F – AGC TGG TCG CGG GTC AGA GGA AGA R – AGT GGT CGC GAT GGG GCC ATG GTG	3.3	700
Ratnaparkhe et al. (1998)	UBC-825	ACA CAC ACA CAC ACT	5.0	1200
Winter et al. (2000)	TA-59	F - ATC TAA AGA GAA ATC AAA ATT GTC GAA R - GCA AAT GTG AAG CAT GTA TAG ATA AAG	3.8	258
	TA-96	F –TGT TTT GGA GAA GAG TGA TTC R- TGT GCA TGC AAA TTC TTA CT	3.3	275
	TR-19	F - TCA GTA TCA CGT GTA ATT CGT R- CAT GAA CAT CAA GTT CTC CA	3.1	227

markers previously reported to linked with disease resistance gene were tested for their ability to differenttiate the wilt resistance and susceptible genotypes. It was observed that two RAPD markers CS-27 and UBC-170 reported by earlier worker linked to disease resistance gave amplification of 700 and 550bp, respectively in susceptible genotype only (Figure 1A and B). The SCAR marker CS-27A gave amplified product of 700 bp in susceptible genotypes only, the amplification was absent in other genotypes (Table 2 and Figure 1C). Further, the ISSR marker UBC-825 gave amplification of 1200 bp in susceptible and moderately susceptible genotypes. Three STMS primer (TA-59, TA-96 and TR-19) were utilized in present study to characterize chickpea genotype for *foc*-4 resistance. The PCR amplification of TA-59 primer generated two alleles, out of which the allele of 258 bp was observed only in resistant genotypes (Figure 1E). The alleles of 265 bp amplified by primer TA-96 was present only in resistant genotypes; whereas the same was absent in other genotypes (Table 2 and Figure 1F). The marker TR- 19 amplified allele of 227 bp in resistant genotypes. The seed storage proteins of chickpea genotypes separated on 10% SDS PAGE resolved a total number of 21 bands (Figure 2). In present study, differences have been observed among studied genotypes based on protein profiling but none of polypeptide fragment was specific to wilt resistance or susceptibility. However, dendrogram based on Nei's similarity coefficient could distinguish some sort of grouping among resistant and susceptible genotypes. Moderately resistant genotype Chaffa was laid on separate cluster while resistant genotype WR-315 was laid on sub-cluster of cluster-I. Moreover, another resistant and tolerant genotype that is, ICCV-2 and GG-1 was laid on same subcluster of cluster-II, the susceptible genotypes GG-4 and JG-62 were present in one cluster (Figure 3). These results may be further confirmed using large number of diverse chickpea accessions.

The seed storage protein profiling by SDS-PAGE had been exploited for inter and intra species diversity analysis in *cicer* (Asghar et al., 2003; Hameed et al., 2009). But it had not been exploited until today for characterization of disease resistance in cicer. Total seed storage protein profiling by SDS-PAGE revealed the presence of two unique protein of ~97 and ~100 kDa in pearl millet genotypes resistant to downey mildew (Mahatma et al., 2011). The molecular markers linked to either susceptibility or resistance have been effectively utilized for MAS. In present study, we have employed four different susceptibility linked markers (namely CS-27, UBC-170, CS-27A and UBC-825) which amplified specific fragment of reported size in susceptible genotypes only. Tullu et al. (1999) reported that the RAPD ($CS\text{-}27_{700}$) marker locus is linked to one of the resistance genes inferred from the F_2 phenotypic data. They found that the marker linked to the *fusarium* wilt resistant genes consistently amplified a distinct DNA fragment (700 bp) in the susceptible F_2 plants. Similar size of fragment was observed in susceptible genotypes (JG 62 and GG 4) in present study. The RAPD marker $CS\text{-}27_{700}$ and $UBC\text{-}170_{550}$ were also reported to link with the susceptibility (Tullu et al., 1998). The study on inheritance and linkage of a gene for resistance

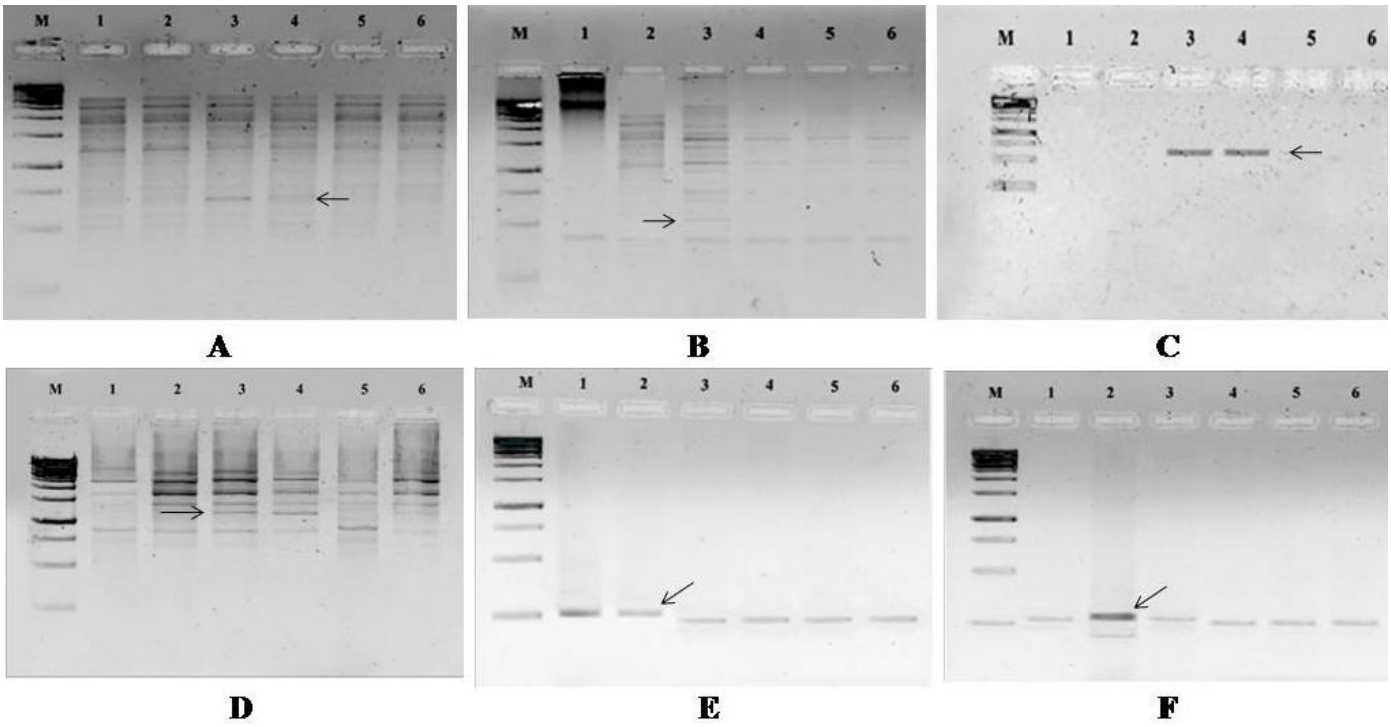

Figure 1. A subset of alleles revealed by primer pairs (A) CS-27, (B) UBC-170, (C) CS-27A, (D) UBC-825, (E) TA-59 and (F) TA-96 among six diverge genotypes of *Cicer arietinum* L. PCR products were separated on agarose gel along with DNA ladder and photographed by gel documentation system (Bio- Rad, USA). Lanes 1 to 6: M = 250 bp DNA ladder, 1 = WR-315, 2 = ICCV-2, 3 = JG-62, 4 = GG-4, 5 = GG-1 and 6 = Chaffa. The fragment of interest (described in Table 2) has been marked with arrow.

Table 2. Reaction of six diverse chickpea genotypes with seven molecular markers linked to disease resistance. The presence of specifically disease resistance/susceptibility linked DNA fragment was analysed using fragment size analysis tool available in Bio- Rad, USA by comparing it with standard DNA ladder.

Genotype	Wilt reaction	Markers linked to the resistance gene (bp)						
		RAPD		SCAR	ISSR	STMS		
		CS-27	UBC-170	CS-27A	UBC-825	TA-59	TA-96	TR-19
WR-315	Resistant	-	-	-	-	258	265	227
ICCV-2	Resistant	-	-	-	-	258	265	227
JG-62	Highly susceptible	700	550	700	1200	-	-	-
GG-4	Susceptible	700	550	700	1200	-	-	-
GG-1	Tolerant	-	-	-	-	-	-	-
Chaffa	Moderately susceptible	-	-	-	1200	-	-	-

to race 4 of *fusarium* wilt and RAPD markers in chickpea shown that these two RAPD markers were located 9 map unit from the race 4 resistance locus and were on the same side of resistance gene. The linkage of the CS 27_{700} marker with wilt susceptibility was established through study on fifteen genotypes with diverse background (Soregaon and Ravikumar, 2010). Ratnaparkhe et al. (1998) identified new ISSR primer (UBC-825_{1200}) by changing the anchored region of the ISSR primers previously reported to linked with disease resistance gene.

The repeat $(AC)_8T$ amplified a marker, UBC-825_{1200}, which was located 5.0 centi Morgan (cM) from the gene for resistance to *fusarium* wilt race 4 and was closer than other markers. The microsatellite based UBC-825 was also able to identify the intermediate reacting genotype. So, considering the ability to give the amplification in intermediate genotypes also, this marker should always be used with other reported markers in order to avoid miss leading conclusions. Three different STMS markers (TA-59, TA-96 and TR-19) linked to resistance allele by 3.8, 3.3 and 3.1 cM distance respectively were shown expected amplification in resistant genotypes only. Winter et al. (1999) characterized and mapped 120 STMS on the chickpea genome map. The primer TA-59, TA-96 and TR-19 were mapped on same linkage group on which gene for disease resistance was present (Winter et al., 2000).

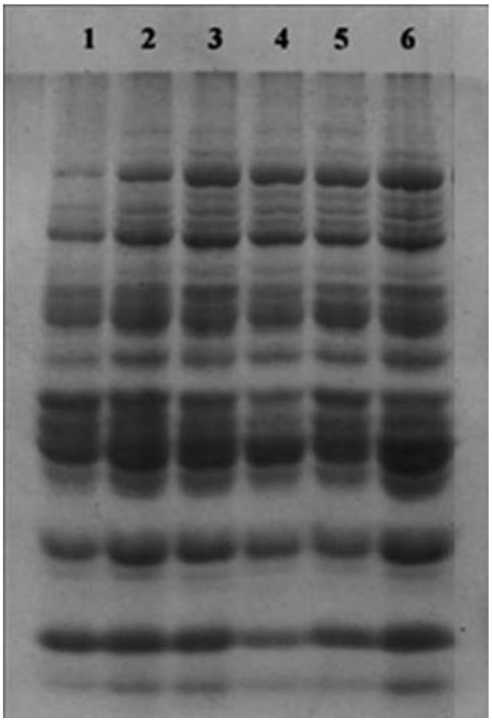

Figure 2. Seed storage protein profile generated by electrophoresis on 10% SDS PAGE. The PAGE resolved total 21 polypeptide fragments, but the fragment specific to susceptibility or resistance was absent. Lanes 1 to 6: 1 = WR-315, 2 = ICCV-2, 3 = JG-62, 4 = GG-4, 5 = GG-1 and 6 = Chaffa.

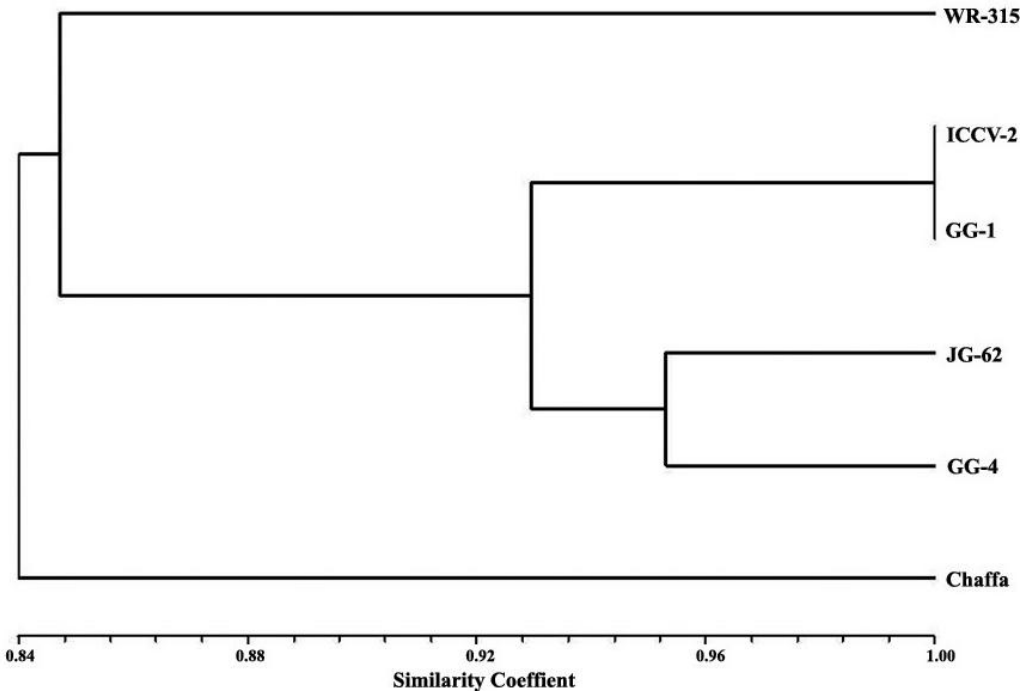

Figure 3. The dendrogram generated by NTSYS-pc (version 2.02i) based on UPGMA using Jaccard's coefficient of seed storage protein data. The dendrogram distinguished some sort of grouping among resistant and susceptible genotypes.

The amplification size of TA-59, TA-96 and TR-19 as characterized in *cicer* sp. were 278, 275 and 227 bp, respectively. Our results are closely consistent with reported results. These three STMS primers showed specific amplification pattern in resistant genotype, which can be effectively utilized for large scale screening in disease resistance breeding as well as for marker assisted breeding programme.

In the present study, seed storage protein profiling and seven different molecular markers (CS-27, UBC-170, CS-27A, UBC-825, TA-59, TA-96 and TR-19) linked to disease resistance were analyzed on six diverse chickpea genotypes. Though, seed protein fragment analysis offered clustering of genotypes, fragment specific to resistance or susceptibility was not reported. The molecular markers validated in this study could be effectively utilized for marker assisted selection in disease resistance breeding of chickpea.

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Full Length Research Paper

DNA polymorphism analysis of *Xanthomonas campestris* pv. *campestris* using single-strand conformation polymorphism (SSCP) and random amplified polymorphic DNA (RAPD)

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The applicability of the random amplified polymorphic DNA (RAPD) and single-strand conformation polymorphism (SSCP) techniques using M13 and 16S rRNA primers, respectively, for genotyping of the phytopathogenic bacterium *Xanthomonas campestris* pv. *campestris* was studied. RAPD provided a simple, rapid, and reliable method to identify genetic variation, and SSCP was used to screen for sequence polymorphisms. A 730-bp region amplified using 16S rRNA primers was subjected to restriction digestion followed by SSCP analysis (restriction fragment length polymorphism (RFLP)-SSCP) to identify polymorphisms, and the results were compared with the RAPD profile obtained using the phage M13 core sequence as a single primer. SSCP is a useful tool for the detection of mutations but large amplicon size can hinder secondary structure formation. Therefore, RAPD using an M13 primer is more efficient for phylogeny detection. The use of a novel polymerase chain reaction (PCR)-based DNA fingerprinting method using both RAPD and SSCP to detect DNA sequence diversity is reported. This approach consists of PCR amplification of the 16S ribosomal DNA with universal primers and analysis of the PCR product by SSCP. This is the first report wherein plant pathogens are subjected to RFLP-SSCP combined with M13 primer-based analysis. This report provides a rapid and reliable method for phylogenetic analysis.

Key words: *Xanthomonas campestris* pv. *campestris,* polymerase chain reaction (PCR), single strand conformation polymorphism (SSCP), microbial phylogenetics, randomly amplified polymorphic DNA (RAPD).

INTRODUCTION

Plant pathogenic bacteria are responsible for increasing economic losses worldwide. They can cause a large range of symptoms in most cultivated plants, and affect different geographic regions with various degrees of agronomic impact. The genus, *Xanthomonas*, is a diverse and economically important group of bacterial phyto-pathogens belonging to the gamma subdivision of the Proteobacteria. *Xanthomonas campestris* pv. *campestris*,

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Abbreviations: SSCP, Single-strand conformation polymorphism; **ssDNAs,** single stranded DNAs; **RAPD,** random amplified polymorphic DNA; **PBS,** phosphate buffered saline; **EDTA,** ethylene diamine tetra acetate; **SDS,** sodium dodecyl sulfate; **RFLP,** restriction fragment length polymorphism; **YDCC,** yeast dextrose calcium carbonate; **HTS,** high-throughput screening; **ARDRA,** amplified rDNA restriction analysis; **DGGE,** denaturing and temperature gradient gel electrophoresis.

a gram-negative aerobic rod, is the causal agent of black rot, which affects crucifers. The symptoms caused by this pathogen include marginal leaf chlorosis and darkening of the vascular tissue, accompanied by extensive wilting and necrosis. Full leaf yellowing, wilting, and necrosis occur as the disease advances. *X. campestris* pv. *campestris* is also grown on a commercial scale to produce the exopolysaccharide xanthan gum, which is used as a viscosifying and stabilizing agent in many industries.

The control of plant pathogens relies on the ability to identify infecting species. However, the traditional DNA–DNA hybridization technique is not suitable for the routine identification of new pathogen isolates, therefore, alternative molecular methods have been developed. Studies of the 16S ribosomal RNA (rRNA) gene (Hauben et al., 1997; Moore et al., 1997a, 1997b) and the 16S–23S intergenic region (Gonçalves and Rosato, 2002) can generally only be used to identify strains at the genus level. Ribosomal DNA (rDNA) is suited for phylogenetic studies because the degree of conservation varies between the different rDNA components. The 16S rRNA sequence, which is highly conserved among species throughout evolutionary history, is found in all prokaryotic organisms and is one of the most extensively studied target sequences. The 16S rRNA gene contains also variable regions, which have been used for discrimination between species and genera. The conserved sequences of the16S rRNA have led to the development of conserved primers for the detection of eubacteria. However, despite the fact that rRNA genes are universal to all living organisms, the correspondence between genome composition and rRNA phylogeny remains poorly characterized.

Nucleic acid-based amplification systems such as polymerase chain reaction (PCR) are promising methods for the rapid detection of low numbers of bacterial cells. Single-strand conformation polymorphism (SSCP) is a rapid and sensitive approach for characterization of DNA sequences. This method, first described by Orita et al. (1989) has been used for the detection of various alterations in DNA sequences including substitutions, deletions, insertions, and rearrangements. SSCP analysis resolves DNA sequences on the basis of the characteristic size and shape of the folded structure, which is determined by the inter-molecular interaction of ssDNA and therefore by the nucleotide sequence. The SSCP technique was initially developed for examining point mutations in human DNA (Orita et al., 1989), and since has been extended to the study of the variability of plant pathogens, including viruses (Kong et al., 2000), nematodes (Clapp et al., 2000) and fungi (Kumeda and Asao, 1996). PCR products are now routinely used for SSCP analysis. After PCR amplification of the target sequence, the amplified product is denatured to two single stranded DNAs (ssDNAs) and subjected to non-denaturing polyacrylamide gel electrophoresis. The mobility of the ssDNA under non-denaturing conditions depends on the secondary structure of the amplified product; ssDNA that is determined by the nucleotide sequence. The ssDNA bands at different positions on the gel indicate different sequences. PCR-SSCP is capable of detecting more than 90% of all single-base substitutions in 200-bp fragments (Hayashi, 1991).

The random amplified polymorphic DNA (RAPD) technique is based on the amplification of genomic DNA with a single primer of arbitrary nucleotide sequence (William et al., 1990; Welsh and McClelland, 1990). The arbitrary primer detects polymorphisms in the absence of specific nucleotide sequence information, and the polymorphisms function as genetic markers. RAPD is a rapid technique to screen for nucleotide sequence polymorphisms, and has been developed to screen for genetic variants. RAPD technology has gained widespread acceptance and application because it is a relatively simple tool for genetic analysis in biological systems. RAPD is generally the preferred assay when the nucleotide sequence is not known. DNA amplification approaches using arbitrary primers have been employed in a large number of genetic studies including the estimation of genetic relationships (Wilde et al., 1992). Furthermore, Kim et al. (1993) reported that PCR has been widely and successfully employed for the DNA sequence diversification analysis of important plant pathogenic bacteria and fungi.

MATERIALS AND METHODS

Isolation of plant pathogenic bacteria

X. campestris pv. campestris was isolated from infected leaf. The other phytopathogenic bacteria used (*Xanthomonas axonopodis* pv. *vesicatoria*, *Ralstonia solanacearum*, and *Xanthomonas oryzae* pv. *oryzae*) originated from Department of Biotechnology, University of Mysore. *Bacillus subtilis* (441) and *Escherichia coli* (K12) were used for the comparative study. All bacteria were subjected to biochemical characterization. The bacterial isolates were subjected to conventional diagnostic tests using standard protocols for cultural, biochemical, physiological, and pathogenic characterization, including a test for the hypersensitive response (Roohie and Umesha, 2012).

Genomic DNA extraction

Genomic DNA was extracted from the following bacteria: *X. campestris* pv. campestris (10 isolates), *X. axonopodis* pv. *vesicatoria, X. oryzae* pv. *oryzae, R. solanacearum, E. coli*, and *B. subtilis.* The total genomic DNA extraction was performed following the protocol described by Gabriel and De Feyter (1992). A loopful of each isolate was suspended in 500 µl of phosphate buffered saline (PBS) in a microfuge tube, mixed by vortexing, and centrifuged at 12000 rpm for 15 min. The supernatant and the viscous material were discarded and the pellet was washed with 1 ml buffer (50 mmol l^{-1} ethylene diamine tetra acetate (EDTA), 0.15 mol l^{-1} NaCl, pH 8.0) and centrifuged repeatedly. Proteinase K to a final concentration of 150 µg ml^{-1} and 30 µl of sodium dodecyl sulfate

(SDS) were added and the suspension was incubated at 50°C for 1 h. An equal volume of a mixture chloroform-phenol-isoamyl alcohol (24:25:1) buffered with 10 mmol l^{-1} Tris-HCl (pH 8.0) was added. The suspension was mixed by vortexing and centrifuged at 12,000 rpm for 5 min to separate the layers. The upper layer was transferred to a fresh microfuge tube and a 0.1 volume of 3 mol l^{-1} sodium acetate and a 1× volume of isopropanol was added and mixed by vortexing. The precipitate was treated with 1 ml of 70% ethanol, and then centrifuged for 2 min at 12000 rpm. The ethanol was subsequently removed and the pellet was resuspended in nuclease-free water. The DNA was quantified using a Nanodrop spectrophotometer (Beckman) and the purity of the DNA was evaluated by electrophoresis in 0.8% agarose gel.

Polymerase chain reaction (PCR) assay

The PCR assay was carried out using 16S rRNA-gene based primer set (Lee et al., 2009) custom synthesized (Sigma Aldrich, USA) with the sequences: 16S-F3 (5′-CCAGACTCCTACGGGAGGCAGC-3′) and 16S-R1 (5′-GCTGACGACAGCCAT GCAGCACC-3′) according to the study of Lee et al. (2009). The PCR amplification was performed with a thermal cycler (LabNet, USA) in a 25 µl reaction mixture containing 1 µl of genomic DNA, 0.35–0.5 µmol l^{-1} of each primer, 0.25 mmol l^{-1} of each deoxynucleoside triphosphate, and 1 µl of reaction buffer (1.5 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} KCl, 10 mmol l^{-1} Tris-HCl, pH 8.8), and 2.0 U of Taq DNA polymerase (Bangalore Genei, India). The following cycling conditions were used: 1 cycle of denaturation for 1 min at 94°C and 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 1 min. The reaction mixtures were stored at 15°C until they were used for analysis. The amplified DNA was detected by electrophoresis in 1% agarose gels (Himedia, India) in 1× TAE buffer (40 mmol L^{-1} Tris-acetate, 1 mmol L^{-1} EDTA, pH 8.0).

16S-SSCP analysis

After the evaluation of the PCR products by agarose gel electrophoresis, 5 µl of an individual PCR product was mixed with 2.5 µl of denaturing buffer (95% formamide, 20 mmol l^{-1} EDTA, 0.05% bromophenol blue). The mixtures were heated at 96°C for 10 min and then chilled on ice. The denatured PCR products (30 µl of each sample) were loaded onto 6% acrylamide–bisacrylamide non-denaturing gels and electrophoresed in pre-chilled 1× TBE buffer at 150 V for 3 h at room temperature. A DNA ladder was loaded to facilitate comparison of the SSCP patterns. After electrophoresis, silver staining of the polyacrylamide gels was carried out according to the method of Bassam et al. (1991). Briefly, the gels were fixed in 10% acetic acid for 20 min at room temperature and then washed with deionized water three times each for 2 min. Silver impregnation was performed for 30 min at room temperature with 0.1% silver nitrate and 0.056% formaldehyde. The gels were then washed for 20 s with deionized water, and color development was performed for 2–10 min with a mixture of 30 g L^{-1} sodium carbonate, 0.056% formaldehyde, and 2 mg L^{-1} sodium thiosulfate. The color reaction was then stopped with 10% acetic acid.

Restriction fragment length polymorphism (RFLP) of SSCP analysis

The restriction enzymes used for restriction fragment length polymorphism (RFLP) analysis in this study included *AlwI, EcoRII*, and *FokI* (New England Biolabs, USA). They were chosen in principle on the basis of the 16S rDNA nucleotide sequences available from *Xanthomonas* sp. databases (http://www.xanthomonas.org/). The amplified rDNA fragment was digested with a restriction enzyme in the buffer recommended by the manufacturer with 6–10 U of enzyme per sample at 37°C for 2 to 3 h with a total volume of 50 µl of PCR product. The reaction was stopped by mixing with the loading buffer described above. The digested PCR products were subjected to electrophoresis in a 2% agarose gel. Each product was also electrophoresed on a 6% polyacrylamide minislab gel as described above.

PCR fingerprinting amplification/RAPD analysis

Genomic DNA from the different strains was used as a template for PCR fingerprinting using a M13 minisatellite core sequence (Huey and Hall, 1989) with the following sequence 5′-GAGGGTGGCGGTTCT-3′. The amplification reactions were performed according to the optimized protocol previously described (Giraffa and Neviani, 2000). One cycle of 94°C for 2 min was followed by 40 cycles of 94°C for 60 s (denaturing), 42°C for 20 s (annealing), and 72°C for 2 min (extension). A final extension was carried out at 72°C for 10 min. The PCR profiles were visualized by electrophoresis in 1.5% agarose gels and staining with ethidium bromide. A 1-kb plus DNA Ladder (Bangalore Genei, India) was used as a DNA molecular weight marker.

Data analysis

Each of the amplification products was identified by its size and number of DNA fragments. The presence or absence of individual bands in the amplified product was scored. A difference matrix was determined using the computer program NTSYS-PC (Applied Biostatistics Inc. CA, USA).

DNA sequencing and sequence analysis

The 16S rRNA gene PCR products were purified and sequenced on an ABI 3730 sequencer (Applied Biosystems, USA). Sequencing was conducted on selected PCR products. Sequences were analysed with the Classifier tool provided by the Ribosomal Database Project (Cole et al., 2009). The 16S sequence obtained in this study has been deposited in GenBank.

RESULTS

Isolation and biochemical classification of bacterial strains

On Tween B medium, *X. axonopodis* pv. *vesicatoria* colonies exhibited typical morphological characteristics such as yellow colonies with zones indicating starch hydrolysis, and *R. solanacearum* colonies–appeared as typical mucoid creamy-white with pink centers. These pathogens were further purified by streaking on the respective semi-selective media. *X. oryzae* pv. *oryzae* colonies showed yellow mucoid shining appearance around the seed and plant material. The isolated bacterial strains showed gram-negative characteristics as determined by both gram staining and KOH solubility testing. All isolated strains were subjected to gram staining, tests

Table 1. Biochemical characterization of *X. campestris* pv. *campestris*, *X. axonopodis* pv. *vesicatoria*, *R. solanacearum*, *X. oryzae* pv. *oryzae* and *E. coli*.

Biochemical test	*Xcc*	*Xav*	*Xoo*	*Rs*	*E. coli*
KOH solubility	+	+	+	+	+
Starch hydrolysis	+	+	+	+	-
Lipase activity	+	+	+	+	-
Xanthomonadin pigment	+	-	+	-	-
Gelatin hydrolysis	+	+	+	+	-
Hypersensitivity to *Nicotiana tobacum*	+	+	+	+	+
Pathogenicity to susceptible Cabbage cv. Golden acre	+	-	-	-	-

All the tests were conducted in replicates and repeated twice. +, positive reaction; -, negative reaction; *Xcc*, *X. campestris* pv. *campestris*; *Xav*, *X. axonopodis* pv. *vesicatoria*; *Rs*, *R. solanacearum*; *Xoo*, *X. oryzae* pv. *oryzae*; *E. coli*, *Escherichia coli.*

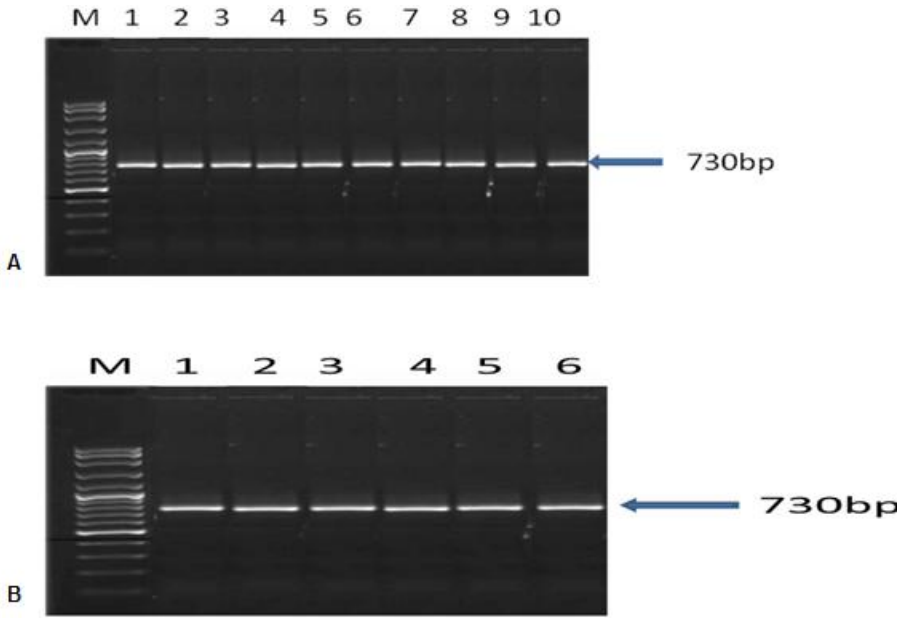

Figure 1. 1A Agarose gel (1%) electrophoresis of PCR products amplified with the 16S rRNA primer set. Lanes: 1 – 10, *X. campestris* pv. *campestris* isolates, Lane M: molecular size markers (100-bp DNA ladder). **1B.** Agarose gel (1%) electrophoresis of PCR products amplified with the 16S rRNA primer set. Lanes: 1 - *X. campestris* pv. *campestris*, Lane 2 - *Xanthomonas axonopodis* pv. *vesicatoria,* Lane 3 - *Xanthomonas oryzae* pv. *oryzae*, Lane 4 - *Ralstonia solanacearum,* Lane 5- *Bacillus subtilis 441,* Lane 6 - *Escherichia coli* K12 Lane M: represents the molecular size marker (100-bp DNA ladder).

for starch hydrolysis, catalase activity, oxidase activity, asparagine medium growth, xanthomonadin pigment production, hypersensitivity to *Nicotiana tabaccum* and pathogenicity to *Brassica oleracea* var *capitata* (cv. Golden Acre) (Table 1).

PCR analysis using the 16S rRNA primer set

Using the 16S rRNA primer set, an approximately 730 bp PCR product was amplified from the genomic DNA of all the 10 isolates of *X. campestris* pv. *campestris*, *X. axonopodis* pv. *vesicatoria*, *R. solanacearum*, *X. oryzae.* pv. *oryzae*, *E. coli,* and *B. subtilis* tested (Figures 1A and 1B).

SSCP analysis

The denatured PCR products amplified by 16S rRNA primer yielded a single conformer. A band shift was observed only in *B. subtilis.* The identical size of PCR product (730 bp) was obtained in all the isolates including

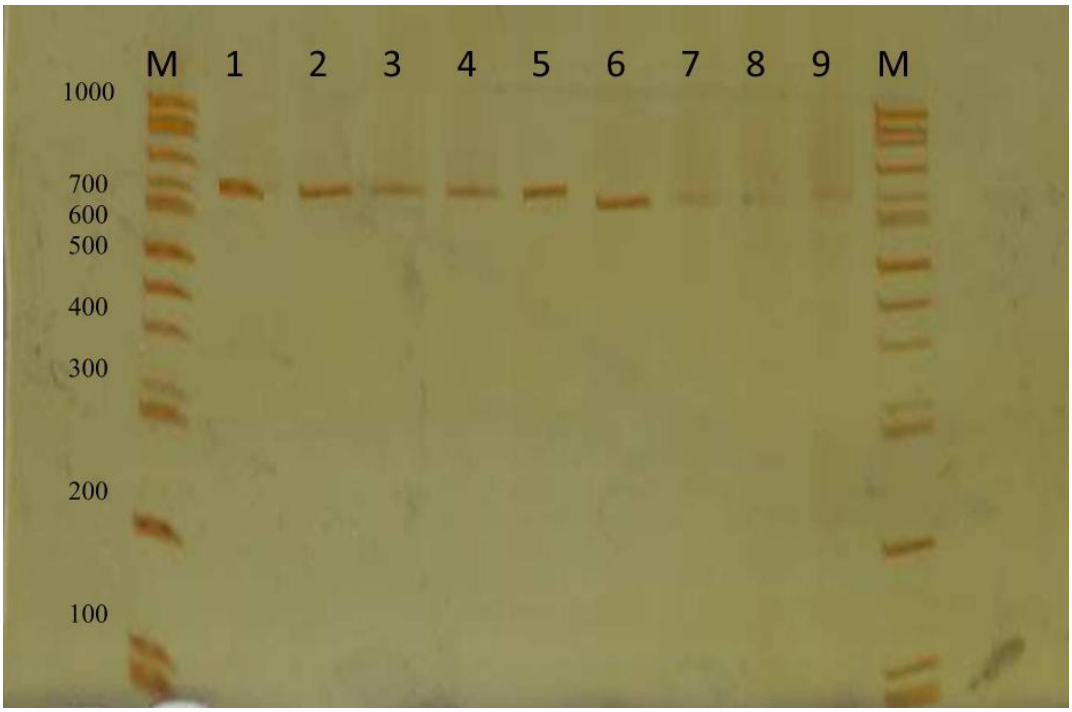

Figure 2. Six percent non denaturing polyacrylamide gel of denatured PCR products amplified with the 16S rRNA primer set. Lane 1, *X. campestris* pv. *campestris*; lane 2, *Xanthomonas axonopodis* pv. *vesicatoria*; lane 3, *Xanthomonas oryzae* pv. *oryzae*; lane 4, *Ralstonia solanacearum*; lane 5, *Escherichia coli*; lane 6, *Bacillus subtilis*; lanes 7, 8 and 9, *X. campestris* pv. *campestris* isolates, Lane M, molecular size markers (100-bp DNA ladder).

the *E. coli* and *B. subtilis* bacteria (Figure 2). Electrophoresis of the denatured PCR amplification product of 730 bp showed only single conformer which was difficult to distinguish among various phytopathogens. This could result from the large amplicon size. Hence the PCR products were subjected to the RFLP analysis followed by SSCP.

RFLP-SSCP

Based on the 16S rRNA sequence from the ribosomal database, the sequences were analysed for the presence of the restriction sites. Only those enzymes which could once cleave were chosen based on the analysis in NEB cutter V2.0 (http://tools.neb.com/NEBcutter2/). The three restriction enzymes viz., *EcoRII*, *AlwI*, and *FokI* were used in the RFLP analyses. The restriction fingerprint obtained by *EcoRII*, *AlwI* and *FokI* digestion was analysed. The *X. campestris* pv. *campestris* gave three bands, *X*. *axonopodis* pv. *vesicatoria* gave two bands, the *X. oryzae* pv. *oryzae* gave five bands, *R. solanacearum* gave three bands, *E. coli* gave three bands and the *B. subtilis* gave a single band. All of them showed a unique banding pattern upon restriction digestion (Figure 3). Each individual band was considered as equivalent independent characters and all the bands were scored as present or absent for each isolate. Banding patterns were converted into binary tables. The data was analyzed using genetic data analysis software, numerical taxonomy and multivariate analysis system (NTSYSpc) version 2.2. A dendrogram was generated using unweighted pair-group method with arithmerical averages (UPGMA).

The dendrograms (Figures 4 and 5) show clustering of all phytopathogens studied based on the RFLP patterns obtained with *AlwI* and *FokI* restrictases, respectively. Four clusters were detected: cluster 1 containing the *Xanthomonas* species (*X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae*), cluster 2 grouped the *X. axonopodis* pv. *vesicatoria* strains, cluster 3 comprising the *R. solanacearum* and cluster 4 *E. coli*. The cluster analysis resulted in grouping the *Xanthomonas* sp. into a prominent cluster compared to other phytopathogens.

As a result of RAPD-PCR analysis of *X. campestris* pv. *campestris*, *X*. *axonopodis* pv. *vesicatoria*, *R. solanacearum*, *X*. *oryzae* pv. *oryzae*, and *E. coli* the distinctive patterns were obtained that allowed to demonstrate a clear differentiation among the species considered in the study. Reproducibility of the RAPD-M13 patterns was assessed by repetition of RAPD analysis. Figure 6 shows the dendrogram based on RAPD patterns of phytopathogens. Five clusters were detected. Cluster 1 contained the *X. campestris* pv. *campestris* and the

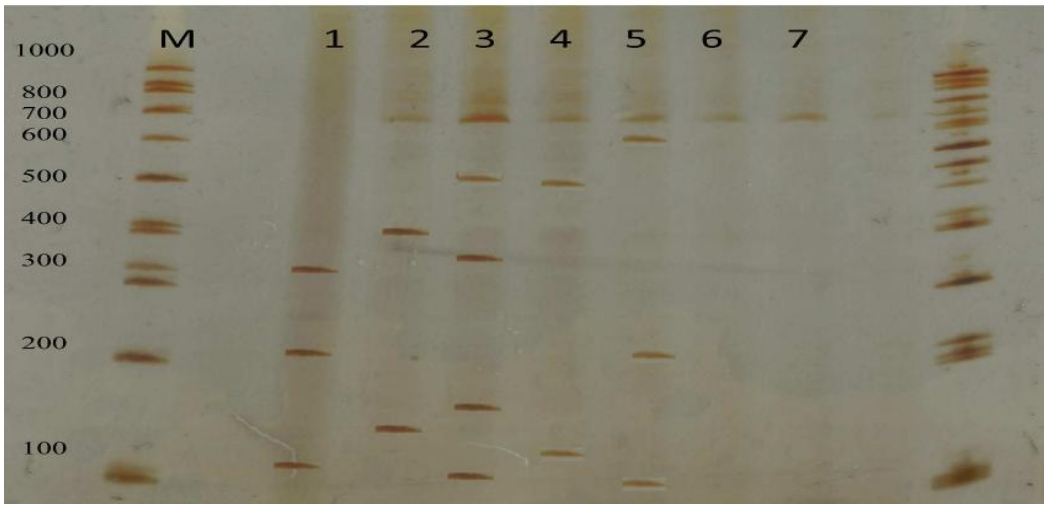

Figure 3. Six percent non denaturing polyacrylamide gel electrophoresis of restriction fragments obtained by 16S rRNA PCR products digested with *EcoRII*. Lane 1, *X. campestris* pv. *campestris*; lane 2, *Xanthomonas axonopodis* pv. *vesicatoria*; lane 3, *Xanthomonas oryzae* pv. *oryzae*; lane 4, *Ralstonia solanacearum*; lane 5, *Escherichia coli*; lanes 6 and 7, *Bacillus subtilis*; lane M, molecular size markers (100-bp DNA ladder).

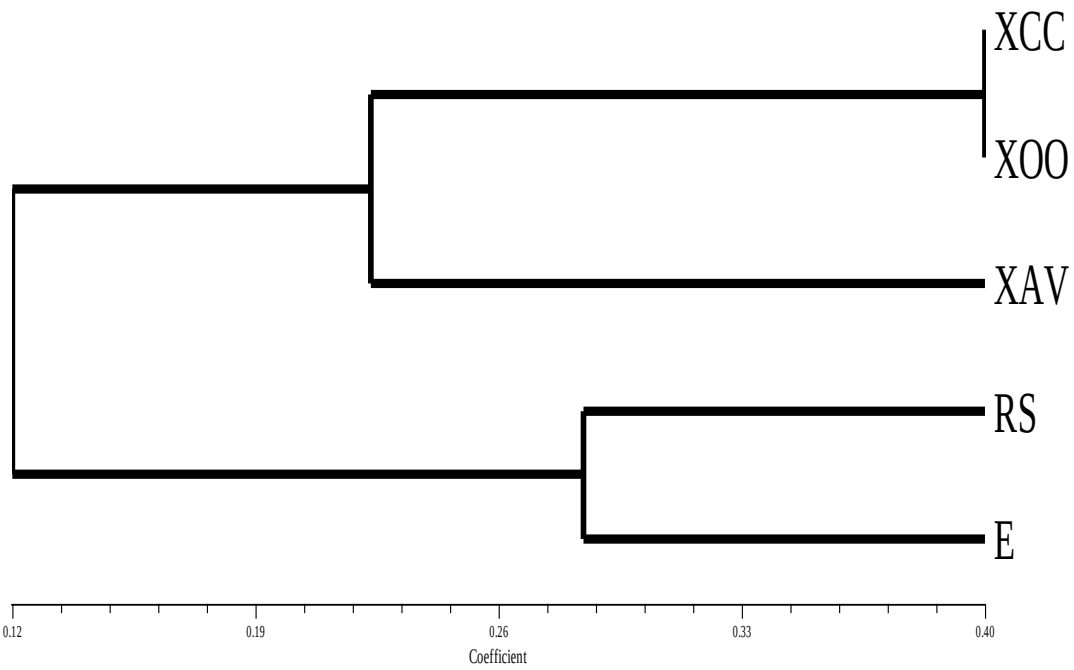

Figure 4. Dendrogram based on the RFLP analysis with *AlwI* restriction enzyme. The tree was obtained by UPGMA cluster analysis. *XCC*, *X. campestris* pv. *campestris*; *XAV*, *X. axonopodis* pv. *vesicatoria*; *RS*, *R. solanacearum*; *XOO*, *X. oryzae* pv. *oryzae*; *E. coli*, *Escherichia coli.*

isolate *X. campestris* pv. *campestris (XCC2).* Cluster 2 consists of the *X. oryzae* pv. *oryzae*. Cluster 3 consisted of the *X*. *axonopodis* pv. *vesicatoria* strains only. *R. solanacearum* was grouped as the fourth cluster and the *E. coli* was grouped into the fifth cluster. The cluster analysis resulted in grouping the *Xanthomonas* sp into a prominent cluster compared to other phytopathogens. The *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* are major *Xanthomonas* sp. producing xanthomonadin pigment. The biochemical characteristics of the

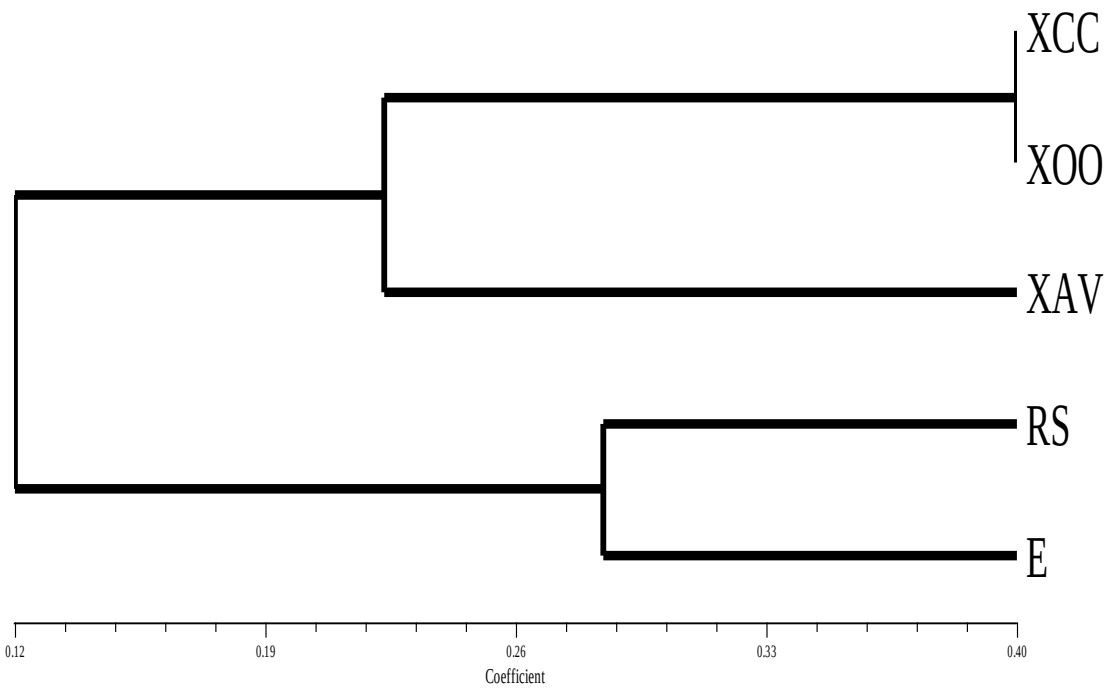

Figure 5. Dendrogram based on the RFLP analysis with *FokI* restriction enzyme. The tree was obtained by UPGMA cluster analysis. *XCC*, *X. campestris* pv. *camp Escherichia coli.*

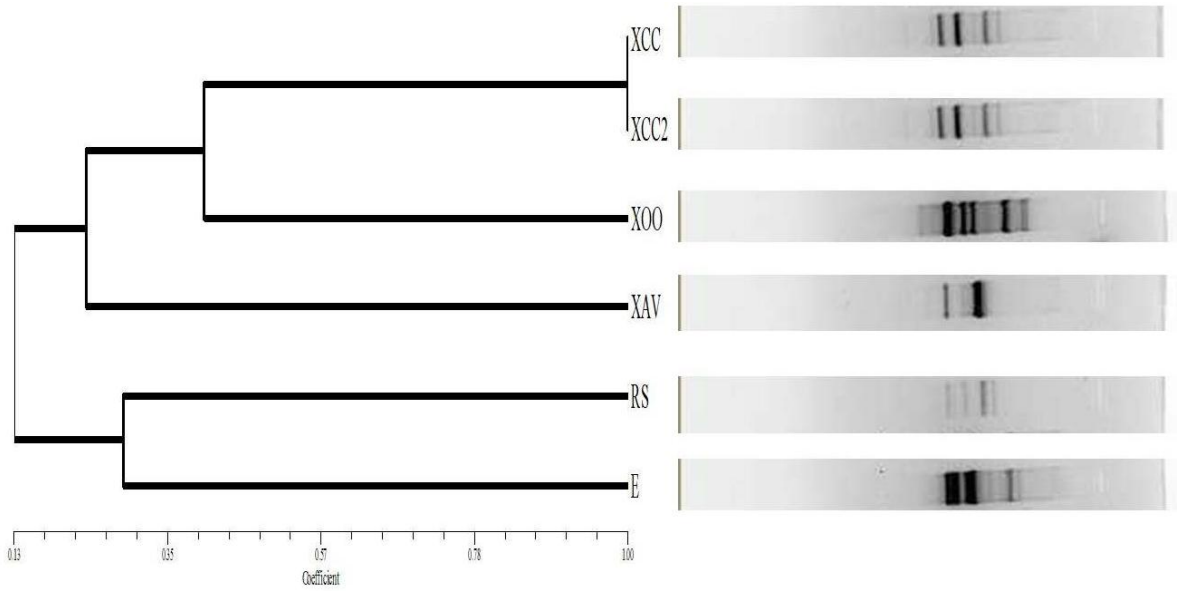

Figure 6. Dendrogram based on the RAPD-M13 profile analysis. The tree was obtained by UPGMA cluster analysis of the RAPD-M13 of various phytopathogens. *XCC* and *XCC2*, *X. campestris* pv. *campestris*; *XAV*, *X. axonopodis* pv. *vesicatoria*; RS, *R. solanacearum*; *XOO*, *X. oryzae* pv. *oryzae*; *E. coli*, *Escherichia coli.* The left part of the figure indicates the dendrogram and the right part the RAPD-M13 banding pattern.

both are similar which is proven by the phylogeny also. The dendrogram obtained by RAPD-M13 (Figure 6) was similar to the dendrograms obtained by *EcoRII*, *AlwI* and *FokI* RFLP analyses. This study confirms the efficacy of RAPD markers. Similar finding has proved the successful application of RAPD markers for estimation of genetic

variability.

Sequencing and analyses

Sequences were edited to exclude the PCR primer binding sites and manually corrected with Chromas 2 (Chromas Version 2.22; w.technelysium.com.au/chromas.html). The sequence data of 16S rRNA gene fragment has been submitted to the GeneBank database with accession number KC855543. *In silico* analyses indicated that the submitted sequence had homology with the 16S rRNA gene sequence of *X. campestris* pv. campestris in the NCBI database. By using M13 core sequence a distinct band was obtained in *X. campestris* pv. *campestris* compared to other pathogens used in the study. Upon the sequencing and bioinformatic analysis it was found a *hrp* (hypersensitive reaction proteins) encoding gene, whereas the major band in *X. axonopodis* pv. *vesicatoria* and *X. oryzae* pv. *oryzae* was have been found to code for methyltransferase gene and peptidyl-asp metallo endopeptidase, respectively (Unpublished data).

DISCUSSION

The currently used protocol for the detection of the pathogens present in seeds uses Fieldhouse-Sasser and mCS20ABN media (Koenraadt et al., 2005). The morphology of cultures is generally checked in subcultures on media, such as yeast dextrose calcium carbonate (YDCC). Classic bacteriological tests, carbon source metabolic fingerprinting (Biolog, Hayward, CA, USA) (Poplawsky and Chun, 1995), fatty acid analysis (MIDI, Newark, DE, USA) (Massomo et al., 2003) and serological tests using polyclonal or monoclonal antibodies (Alvarez et al., 1994) have been used to speed up the identification of the organisms. All of these methods rely on the availability of databases with the results obtained with representative isolates of different species and pathovars. However, frequent problems with the standard isolates used (for example, misidentification) can complicate the interpretation of new results. The inoculation of susceptible brassica seedlings is still the most reliable method as it provides the ultimate confirmation of the identified pathovar. However, all of these methods are time consuming and inadequate for high-throughput screening (HTS). The identification of *X. campestris* pv. *campestris* at the pathovar level is generally based on the isolation of the pathogen using semi-selective media.

Molecular diagnostics play an increasingly important role in the rapid detection and identification of pathogenic organisms. The genetic variation of ribosomal genes in bacteria offers an alternative to culturing for the detection and identification of these organisms. The genes, such as 16S rRNA gene demonstrated conserved sequence regions ideal for prior targeting. Since each bacterial species has a unique 16S rRNA sequence, all organisms can be differentiated from each other using PCR-SSCP. For instance, 16S rDNA fragments with different sequen-ces may co-migrate, thus generating bands at the same position in the gel. The study of microbial communities has become common in microbial ecology, with the 16S rRNA gene being the most frequently used phylogenetic marker.

In the present study, a 730 bp amplicon was obtained by 16S rRNA primer, which gave a single conformer in all the bacteria studied except a band-shift in one of them. In our study, the SSCP could not yield multiple conformers and hence, we decided to accomplish the restriction digestion of the 730 bp amplicon. SSCP has been reported to lose sensitivity for mutation detection when the fragment size is above 300 bp. RFLP can be considered as another alternative because of the fact that the restriction digestion of the amplicon in RFLP profiles yielded a distinct fingerprint for each of the bacteria. We developed a molecular method for directly determining the identity of the bacteria based on the principle of SSCP of a PCR-amplified DNA fragment. The target DNA sequence was the 730 bp fragment of the 16S rRNA sequence obtained by using conserved primers. We made an attempt to detect DNA sequence among *Xanthomonas* sp. using PCR-based DNA fingerprinting method including both RAPD and SSCP. Species-diagnostic markers should be established from DNA segments exhibiting low genetic polymorphism within a particular species, but showing high genetic divergence between different species. Overlapping patterns (in this case, PCR-RFLP and SSCP) between different species should not be observed. The size of PCR amplicon obtained in our study was 730 bp which is large for being detected by SSCP, hence we decided to perform RFLP and RAPD-M13. Techniques that use enzyme digests such as amplified rDNA restriction analysis (ARDRA), RFLP, and SSCP produce multiple bands for single species, making patterns difficult to evaluate further. Hence we combined SSCP-RFLP and RAPD-M13 to overcome these drawbacks.

Our main focus was to use two molecular typing tools to generate DNA profiles that could help distinguish the *X. campestris campestris* from other *Xanthomonas* species. We used microsatellite typing, which involves the amplification of satellite sequences, short (usually <10 bp) tandem repetitive DNA sequences dispersed throughout the genome (Perez et al., 1998). The method relies on the significant level of polymorphism in the lengths of the microsatellite loci, and has been previously reported generating distinguishing profiles in yeast. Amplification of DNA with a single 10 base-long primer of arbitrary DNA sequence is a PCR-based technique, which yields a series of discrete fragments. This method reveals large number of polymorphisms, which can be used as genetic

markers in research involving species diagnostics, population differentiation and genetic fingerprinting. It is known that the region is highly conserved at the intra-species level, but very variable at inter-species level and has proved to be a useful tool for the diagnosis of closely related species.

The analyses indicated that they have, in general, a higher number of exact matches to the 16S rRNA gene sequences from members of the target group of bacteria. The enzyme restriction mapping disclosed the apparent sequence difference of 16S rRNA gene among them. This study also indicated that RFLP-SSCP would be more reliable compared to SSCP when the amplicon size is >600 bp. The cluster analyses obtained for both of the restriction enzymes used in the study *viz., AlwI* and *FokI* gave a similar clustering pattern with the RAPD-M13 primer. PCR amplification with M13 core sequence proved to be a useful method for discrimination at the species level of *Xanthomonas* genus. We confirmed the applicability of M13 primer for the identification of *Xanthomonas* sp.

The technique, which is rapid and easy to perform, could be an alternative to the conventional approach. Furthermore, in order to design new diagnostic primers, more effective in genetic discrimination among genotypes, discriminate bands could be cloned and sequenced. The alternative methods to study the genomic diversity like DNA microarrays and DNA hybridization are accurate in low diversity systems and expensive. The denaturing and temperature gradient gel electrophoresis (DGGE) results in a pattern of bands in which each band may represent more than one species because of comigration. Compared to these methods the combination of SSCP and RAPD-M13 core sequence prove to be an efficient method to detect diversity.

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Full Length Research Paper

Aphid resistance in florist's chrysanthemum (*Chrysanthemum morifolium* Ramat.) induced by sea anemone equistatin overexpression

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Florist's chrysanthemum (*Chrysanthemum morifolium* Ramat.) belongs to the Asteraceae family and represents the second most important floricultural crop in the world. Most genotypes are sensitive to aphids and infestations can lower quality and cause transmission of viruses. The protease inhibitor Sea Anemone Equistatin (SAE) carries three domains responsible for the inhibition of both cysteine and aspartic proteases. Artificial diet bioassays showed that SAE is readily toxic when ingested by the pea aphid, *Acyrthosiphon pisum*, and the cotton aphid, *Aphis gossypii*. We transformed chrysanthemum genotype 1581 by *Agrobacterium tumefaciens*-mediated transformation with the SAE gene under the control of the chrysanthemum RbcS promoter to induce aphid resistance. Non-choice leaf disk and whole plant bioassays were carried out to analyze deleterious effects of SAE on population growth and survival of both *Myzus persicae* and *A. gossypii*. After 7 days, *M. persicae* populations on specific transgenic lines were up to 69% smaller relative to control populations in a whole plant bioassay. The mortality of cotton aphids was 11% on control lines and up to 32% on transgenic lines after 5 days. The results show that SAE may be a promising agent for the control of some aphid species in transgenic plants.

Key words: *Chrysanthemum morifolium*, aphid resistance, RbcS promoter, sea anemone equistatin, agrobacterium transformation.

INTRODUCTION

Cultivated chrysanthemum (*Chrysanthemum morifolium* Ramat), also classified as *Dendranthema* × *grandiflora* (Anderson, 1987), belong to the Asteraceae family (Salinger, 1991). Commercially, it is known as florist's

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Abbreviations: BAP, 6-Benzylaminopurine; **IAA,** indole acetic acid; **MS,** Musrashige and Skoog's medium; **RbcS,** ribulose-1, 5-bisphosphate carboxylase; **NPTII,** neomycin phosphotransferase; **SAE,** sea anemone equistatin; **PCR,** polymerase chain reaction; **qRT-PCR,** quantitative reverse transcriptase polymerase chain reaction; **LB,** luria-bertani; **TSWV,** tomato spotted wilt virus.

chrysanthemum or autumn queen and predominantly sold as cut flowers in many countries of the world (Erler and Siegmund, 1986). After rose, it is globally the most important floricultural crop (Visser et al., 2007). The wide spectrum of colors and shapes, excellent vase life, and their ability to produce desired grades and types at anytime during the year promoted their economic importance. Control of the main insect pests, various aphids (chrysanthemum aphid *Macrosiphoniella sanborni* Gilette, peach aphid *Myzus persicae* Sulzer, pea aphid *Acyrthosiphon pisum* Harris, and cotton aphid *Aphis gossypii* Glover), thrips (mainly *Frankliniella occidentalis* Pergande) and spider mites (*Tetranychus urticae* Koch) on vegetative and flowering chrysanthemum is a critical problem. Insects often develop resistance against frequently used pesticides, many pesticides are banned in important production areas, and there is a high cost to the application of pesticides (Visser et al., 2007). Although, desirable traits have been introduced using classical breeding, there are severe limitations to this technique in a hexaploid species like (cultivated) chrysanthemum. As an alternative, traits have been introduced by means of *Agrobacterium*-mediated transformation (Fukai et al., 1995; Jaime and Teixeira, 2005; Mao et al., 2011) including practical characteristics relating to biotic resistance against aphids, beet army worm, botrytis and tomato spotted wilt virus (TSWV) (Kim et al., 2011; Visser et al., 2007).

Compared to conventional breeding, genetic engineering offers the advantage that a single gene can be inserted in the background of an established cultivar, and it is possible to stack multiple genes for various traits into a single cultivar (Visser et al., 2007). The search for plant compounds that are suitable for genetically based aphid control has resulted in a wide variety of candidate phytochemicals. Methyl ketones, sesquiterpene carboxylic acids and acyl-glucose esters are examples of compounds from trichomes with toxic properties against aphids (Goffreda et al., 1990). Some plant lectins or agglutinins are toxic to sap-sucking insects (Rahbé et al., 1995). *Galanthus nivalis* agglutinin (GNA) (Stoger et al., 1999; Nagadhara et al., 2003), *Pinellia ternate* agglutinin (PTA) (Yao et al., 2003), concanavalin A (ConA) (Gatehouse et al., 1999) and a lectin from *Amaranthus caudatus* (ACA) (Rahbé et al., 1995) are examples of compounds that significantly inhibit the population development of aphids. Wu et al. (2006) overexpressed the *aca* gene (*A*. *caudatus* agglutinin) in cotton plants under the control of a phloem-specific promoter. Bioassays using cotton aphid showed that most transgenic plants significantly inhibited population growth of aphids. Aharoni et al. (2003) reported that leaves of transgenic plants of Arabidopsis constitutively expressing a dual linalool/nerolidol synthase in the plastids (FaNES1) produced linalool and its glycosylated and hydroxylated derivatives. In dual-choice assays with *M*. *persicae*, the FaNES1-expressing lines significantly repelled the aphids. Similarly, the expression of heterologous protease inhibitors in plants has been used as an approach to induce plant resistance to insects (Ryan, 1990). Protease inhibitors (PIs) are proteins that form complexes with gut proteases and inhibit their proteolytic activity. Plants utilize PIs for defense to moderate the adverse effects from attacking herbivores or pathogens (Christou et al., 2006; Ferry et al., 2006; Zavala et al., 2008; Jongsma and Beekwilder, 2011). Many host plant-derived inhibitors are unsuitable; however, due to the fact that many insects have evolved resistance (Jongsma and Beekwilder 2011). Extensive screening of non-host plant-derived inhibitors has resulted in novel candidate proteins such as SAE that demonstrated promising levels of resistance *in vitro* and *in vivo* against various insect pests including aphids (Gruden et al., 1998; Outchkourov et al., 2004a, 2004b; Ceci et al., 2003).

In contrast to leaf-eating insect pests, aphids are exclusively feeding on plant phloem-sap, which usually displays a high free/bound amino acid ratio. Consequently, they are thought not to rely on extensive protein digestion for their nitrogen supply. Nevertheless, a significant number of PIs was shown to promote deleterious effects on different aphid species (Rahbé et al., 1995; Casaretto and Corcuera, 1998; Quillien et al., 1998; Ceci et al., 2003). Also, a modified Oryza cystatin-I (Oc-IDD86) and a chicken egg white cystatin were shown to reduce the growth and survival of nymphs of *M*. *persicae* in artificial diet assays (Cowgill et al., 2002). The cystatin from rice, OC-I, caused growth reductions of up to 40% and reduced fecundity in pea aphid (*A*. *pisum*), cotton aphid (*A*. *gossypii*) and peach potato aphid (*M*. *persicae*) when fed at levels up to 0.25 mg ml^{-1} (Rahbé et al., 2003a). Serine PIs also show insecticidal effects towards aphids. For example, a systematic study of isoforms of Bowman-Birk type PIs from pea seeds showed varying anti-metabolic effects, including significant mortality, to pea aphid, which were associated with inhibitory activity towards pea aphid chymotrypsin (Rahbé et al., 2003b). Ceci et al. (2003) could identify a novel MTI-2 anti-chymotrypsin inhibitor, Chy8, with highest affinity for bovine chymotrypsin that was highly toxic to nymphs of the pea aphid *A*. *pisum*, and moderately toxic to nymphs of *A*. *gossypii* and *M*. *persicae*.

Equistatin is a protease inhibitor from the sea anemone *Actinia equina* that consists of three thyroglobulin type I domains. The first N-terminal domain acts as a cysteine protease inhibitor, and the second as an aspartic protease inhibitor, while the function of the third domain is not yet known (Strukelj et al., 2000). The protein was found to be highly active against the gut proteases of Colorado potato beetle (CPB; *Leptinotarsa decemlineata* Say), and on artificial diets, the protein induced high mortality of CPB larvae (Gruden et al., 1998). In potato leaves, accumulation of only partly functional equistatin

up to levels of 7% of total soluble protein was achieved (Outchkourov et al., 2003b). Co-expression of cystatins promoted the toxic effects against western flower thrips (Outchkourov et al., 2004a, 2004b). In this study, we examined the deleterious effects of purified equistatin on population growth and mortality of pea, peach and cotton aphids using artificial diet bioassays, and then tested peach and cotton aphid resistance in chrysanthemum plants overexpressing sea anemone equistatin in both leaf disk and whole plant bioassays.

MATERIALS AND METHODS

Plant material and insects

Sterile florist's chrysanthemum cultivars 1581 (Plant Research International) were propagated on MS (Murashige and Skoog, 1962) medium containing 3% sucrose and 0.8% agar (w/v). Internode explants were used for transformation. For artificial diet assays, pea, cotton and peach aphids were obtained from established colonies at Biologie Fonctionelle Insects & Interactions (Lyon, France) maintained on *Vicia faba*. For plant bioassays, peach and cotton aphids were obtained from established colonies on Chinese cabbage at the Departments of Entomology and Biointeractions (Wageningen University and Research Center, The Netherlands). Transgenic plants were grown in the greenhouse with supplementary high-pressure sodium light under 16/8 h light/dark rhythm and temperature regime of 21/18°C, and used for molecular and biochemical analyses as well as bioassays.

Artificial diet bioassays

The effects of purified recombinant equistatin was tested against the pea aphid (*A*. *pisum*), peach aphid (*M*. *persicae*) and cotton aphid (*A*. *gossypii*) to determine the toxicological indices of recombinant SAE towards these aphids according to the methods described by Rahbé et al. (2003a). Aphids were weighed on a Setaram (Lyon, FRA) analytical microbalance at the nearest microgram, and bioassays were carried out as fully described previously (Rahbé and Febvay, 1993). Recombinant equistatin was produced and purified as described by Outchkourov et al. (2002). The protein sample was diluted in a stock solution of the aphid diet and water was added to reach the desired concentration. A control diet was prepared in the same way using the buffer of the protein sample.

Transformation and regeneration

Internodes of chrysanthemum cultivar 1581 were pre-cultured on regeneration medium (MS supplemented with 1.0 mg l^{-1} BAP and 0.1 mg l^{-1} IAA) for 2 days. The highly virulent *Agrobacterium tumefaciens* strain AGL-0 with the binary vector pBINPLUS and Rubisco small subunit promoter driving the native equistatin (EIM) gene (pUCRBC-EIM) was used in this experiment (Outchkourov et al., 2003b). A single colony was cultured in 5 ml liquid LB containing 50 mg l^{-1} Kanamycin and Rifampicin and grown at 28°C on a shaker overnight. The culture was diluted 1/100 in fresh LB medium with the same antibiotics and was used for transformation after overnight culture at 28°C. Internodes with a length of 3 mm were cut by scalpel and kept on regeneration medium for 2 days.

Pre-cultured internodes were then collected in 30 ml liquid MS and then 0.6 ml Agrobacterium culture with 30 µl acetosyringone (0.1 M) were added and incubated for 30 min. Explants were then first transferred to sterile filter paper for a few minutes to remove excess bacteria and then co-cultured on solid regeneration medium with 100 µM acetosyringone in darkness at 25°C for 2 days. After co-culture, the explants were transferred to selection medium (regeneration medium containing 400 mg l^{-1} vancomycine, 250 mg l^{-1} cefotaxime and 30 mg l^{-1} kanamycin) and incubated under light for selection. All explants were transferred to fresh selection medium every 21 days and maintained for 65 days after inoculation.

Regenerating shoots were induced from Agrobacterium-inoculated explants 2 weeks after applying antibiotic selection, and reached a final transformation frequency of 8.75%. Green regenerated shoots were transferred to rooting medium (½ MS supplemented with 200 mg l^{-1} vancomycin and 125 mg l^{-1} cefotaxime). Rooted plants were transferred to the greenhouse after being hardened and *Agrobacterium*-free test. Transformation frequency was calculated as the number of PCR-positive greenhouse plants divided by the number of inoculated explants.

DNA and RNA analysis

Genomic DNA was isolated from young leaves as described by Pereira and Aarts (1998) and was used primarily for PCR screening. Primers with product size of 100 bp were designed to identify the EIM sequence (EIM-Forward: GATGTTCGTGCCAGAGTGTT; EIM-Reverse: TCGGAACCTGGAACCTTTAC) using website http://www.genscript.com for use in both PCR and qRT-PCR analysis. A volume of 4 µl DNA was used for PCR, adding 0.5 µl superTaq polymerase and 2.5 µl of 10× buffer, 1 µl of 10 mM specific forward and reverse primers, 0.25 µl of 10 mM dNTP and water to a final volume of 25 µl. Amplification was performed in the GeneAmp PCR system at the following conditions: 94°C, 5 min, 35 cycles of 94°C, 30 s and 55°C, 30 s; 72°C, 20 s; then finally 72°C, 7 min with a drop to 4°C). Positive lines were analyzed by qRT-PCR to determine the level of gene expression. Total RNA was extracted by the TriPure^{TM} small sample method. cDNA synthesis was done using the TaqMan^{TM} Reverse Transcription Reagents. Reverse transcription was performed in the GeneAmp PCR system at the following conditions: 25°C for 10 min; 48°C, 30 min; 95°C, 5 min. A volume of 1 µl of cDNA (2 µg) was used for qPCR, with 10 µl BIO-RAD iQ^{TM} SYBR$^{@}$ Green Supermix, 2 µl of 3 µM specific forward and reverse primers, and a volume of 20 µl water. The housekeeping gene actin was used as reference gene (Actin-Forward: CCTCTTAATCCTAAGGCTAATCAG; Actin-Reverse: CCAGGAATCCAGCACAATACC). Amplification and real-time measurements were performed in the iCycler iQ5 (Bio-Rad, USA) (95°C, 3 min, 40 cycles of 95°C, 10 s and 60°C, 30 s; 95°C, 1 min; 60°C, 1 min).

The results were analyzed using the IQ5 Optical System Software and $2^{-\delta Ct}$ CT Method (Livak and Schmittgen, 2001).

Western-blot analysis

A piece of a fully expanded young leaf (200 to 300 mg) was placed in a 1.5 ml eppendorf tube and ground in liquid nitrogen to a fine powder. The powder was re-suspended in 300 µl of extraction buffer [100 mM Tris-HCl, pH 7.6, 25 mM sodium-diethyldithiocarbamate, 50 mM EDTA, 10% (w/v) polyvinylpolypyrrolidone (PVPP), and 1 tablet 25 ml^{-1} protease inhibitors cocktail complete (Prod. Nr. 04693159001, Roche Diagnostics)], and this crude extract was twice centrifuged for 5 min at 14,000 rpm and 4°C. Each time, the supernatant was replaced into a new tube. SDS-PAGE was performed using a 15% (w/v) precast resolving gel

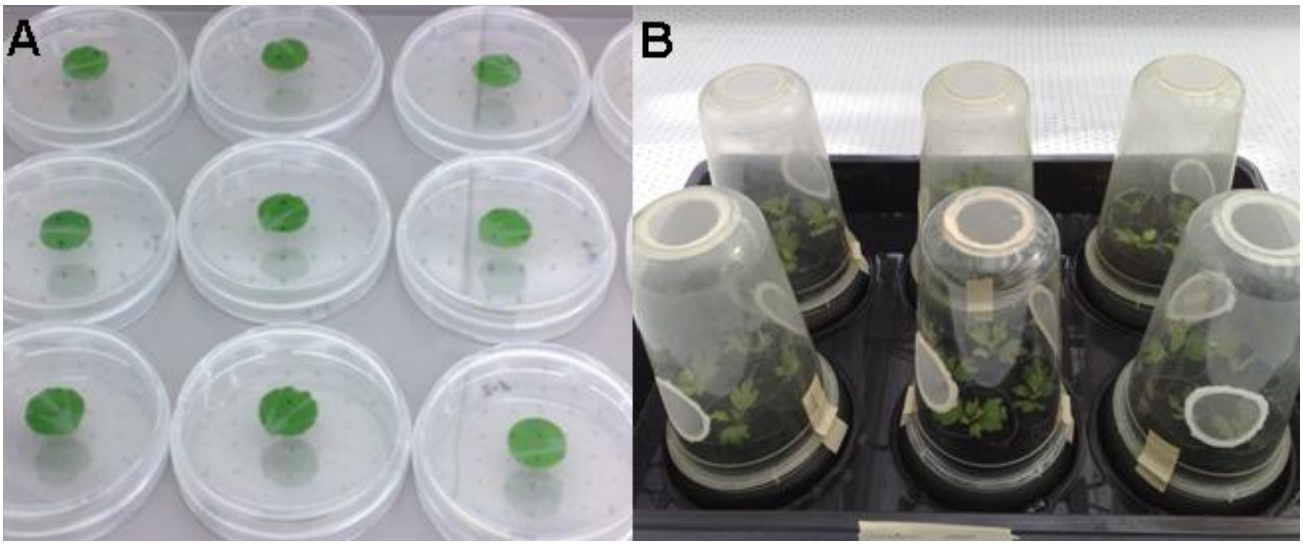

Figure 1. Non-choice leaf disk bioassay A), Whole plant bioassay B).

(Bio-Rad Laboratories) on a mini-Protean II slab cell apparatus (Bio-Rad Laboratories). The gels were run according to the manufacturer's instructions. The separated protein samples from the SDS-PAGE gels were transferred to Trans-Blot (Bio-Rad Laboratories) nitrocellulose membranes using the mini-Protean II electro-transfer apparatus (Bio-Rad Laboratories). Subsequently, the membranes were blocked in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% (v/v) Tween 20 containing 2% (w/v) non-fat milk powder for 1 h and then incubated with rabbit anti-EI antibodies (Eurogentec, Seraing, Belgium). The blots were subsequently washed and incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA).

The membranes were visualized with Lumi-Light western blotting substrate and scanned in the Lumi-Imager F1 under the control of Lumi-Analist software (Roche Diagnostics, Mannheim, Germany).

Plant bioassays

Non-choice leaf disk and whole plant bioassays were carried out to test the effects of equistatin expression on population development and mortality. Adult aphids of both species were collected with a fine brush from a rearing on Chinese cabbage. For non-choice bioassays, a single 2 cm leaf disk from mature green leaves of transgenic or wild type chrysanthemum plants were placed abaxial side up on a thin layer of 1% water-agar in a Petri dish with 9 cm diameter (Figure 1A). Five separate Petri dishes were prepared as replicates for each transgenic line examined. Five aphids were released in the lid of a Petri dish and, after closing, the Petri dishes were incubated upside down at 20°C under long-day conditions (16 h of light/8 h of dark) with a RH of 70%. The numbers of aphids on each leaf disk were recorded at a range of time points after the start of the experiment. For whole plant bioassays, young plants were used in cages containing four replicates for each line (Figure 1B). Each plant was inoculated with ten aphids and the number of aphids on each plant was counted after 1 week. All data were analyzed by SAS v.7.0. Analysis of variance (ANOVA) was used to compare different lines and the significant difference of the means was calculated using Duncan's multiple range test (P = 0.05).

RESULTS

Artificial diet bioassays

To test the activity of equistatin artificial diet, bioassays were carried out against pea, peach and cotton aphids, which are known to colonize chrysanthemum plants. Artificial diet bioassays showed that SAE is readily toxic when ingested by the pea aphid, *A*. *pisum* [IC50 ≈ 150 µg ml^{-1} (10 µM); LC50 ≈ 190 µg ml^{-1} (13 µM)], and the cotton aphid *A*. *gossypii* [IC50 ≈ 870 µg ml^{-1} (58 µM); LC50 ≈ 203 µg ml^{-1} (14 µM)], while it showed only moderate growth inhibition of the peach aphid *M*. *persicae* (Figure 2). Equistatin at a concentration of 482 µg ml^{-1} caused 75 to 80% mortality of pea and cotton aphid (Figure 3), but there was no significant effect on survival of peach aphid (not shown).

Molecular and biochemical analysis of transgenic plants

The result of PCR for some transgenic lines is shown in Figure 4. Subsequently, gene expression levels were analyzed by qRT-PCR using the same primers as used in PCR. Based on the number of PCR cycles, the relative transcript levels were estimated to be in the range of the native Rubisco small subunit gene (data not shown). The expression level of the SAE gene ranged in 7 independent transformants 10-fold, and was highest in line 15-4 (Tables 1 and 2). The expression of equistatin protein was demonstrated by western blot of SDS-PAGE gels of extracted leaf protein. Four bands were assigned to SAE as they were absent in the control. There was no

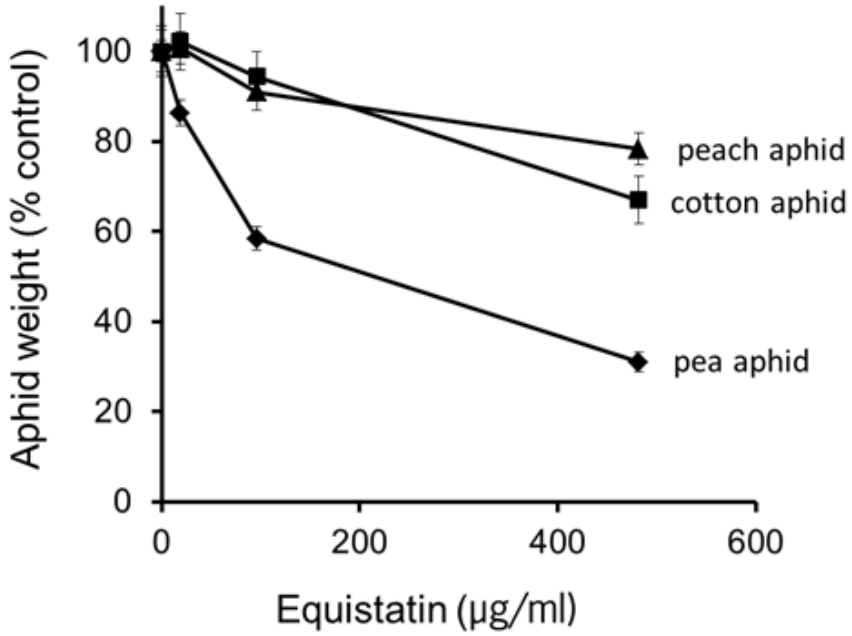

Figure 2. The effects of recombinant SAE protein on the growth of pea, peach and cotton aphids in artificial diet experiments.

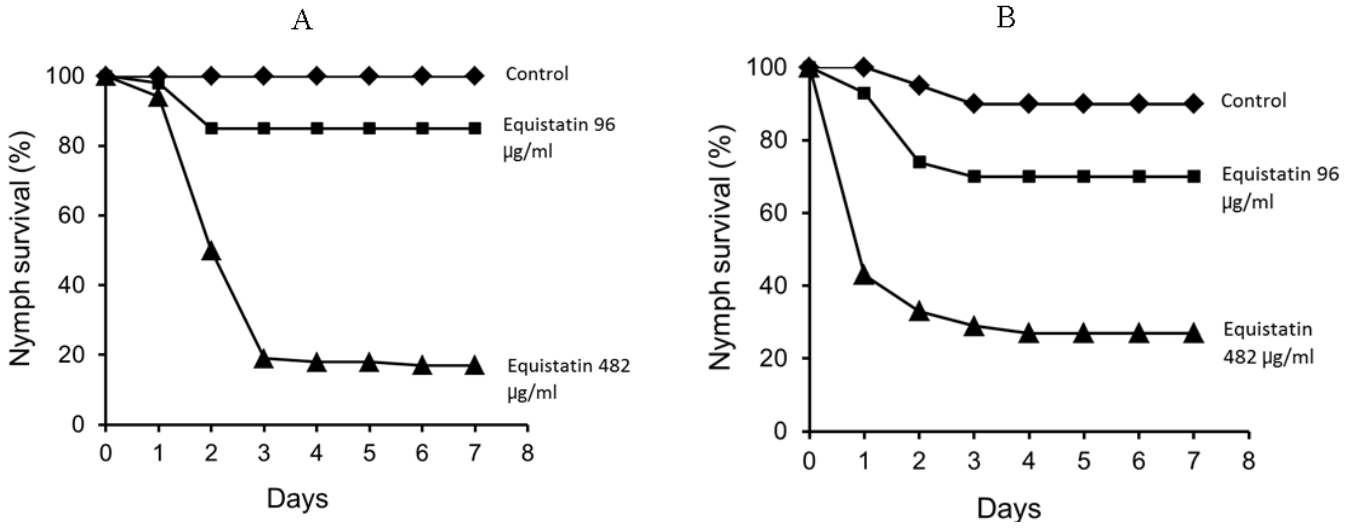

Figure 3. The effects of recombinant SAE protein on survival of pea (A) and cotton (B) aphids in artificial diet experiments.

obvious correlation of the western blot results with the qRT-PCR based ranking of expression levels. If we took samples at the end of the day, our gels did not reveal any protein (Figure 5).

Plant bioassay

To test the effectiveness of SAE gene expression in controlling aphid populations, various bioassays on leaf disks and whole plants were carried out with both peach and cotton aphids. In non-choice leaf disk bioassays with peach aphids, like in the artificial diet, no increase in mortality was observed, but the growth of the peach aphid population was strongly inhibited from a 120% increase on wild type chrysanthemum plants to only 8% on line 15-4 with the highest expression level. Intermediate expression levels demonstrated growth reductions that correlated well with the expression level as measured by qRT-

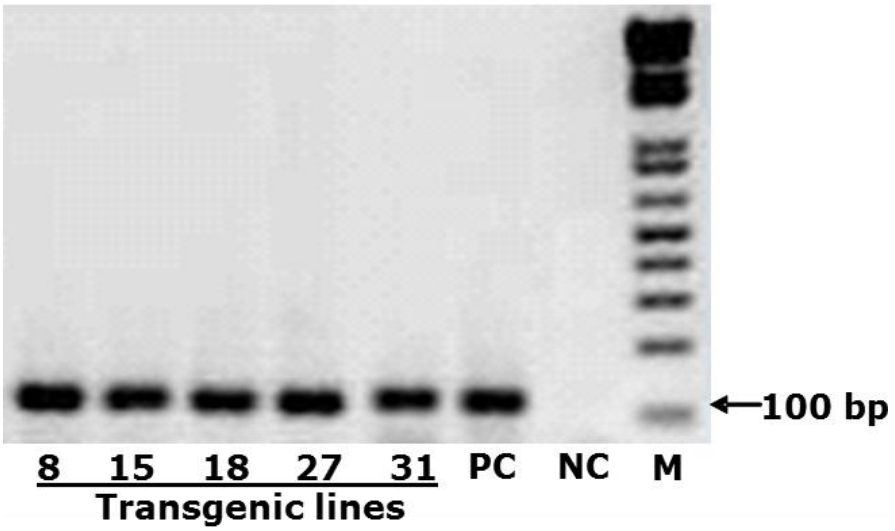

Figure 4. PCR analysis for the presence of the SAE gene in Agrobacterium negative transgenic chrysanthemum plants from the greenhouse. M: size marker, PC: positive control, NC: negative control.

Table 1. Population development of peach aphids (*Myzus persicae*) in no choice leaf disk and whole plant bioassays of chrysanthemum 1581 transformed with the SAE gene.

Line	SAE gene expression	No choice leaf disk bioassay (5 nymphs, 5 days, n = 5)		Whole plant bioassay (10 aphids, 7 days, n = 4)	
	qRT-PCR (%)	Average number	% increase	Average number	% increase
15-4	100.0^{a}	5.4 (±0.24)^{b}	8^{b}	27.7 (±9.41)^{b}	177^{b}
31-1	71.0^{a}	Nt	Nt	20.7 (±3.97)^{b}	107^{b}
27-2	33.7^{b}	6.0 (±0.77)^{b}	20^{b}	36.2 (±6.91)^{ab}	262^{ab}
18-4	33.7^{b}	Nt	Nt	50.2 (±12.81)^{ab}	402^{ab}
8-2	11.2^{c}	8.4 (±1.53)^{ab}	68^{ab}	36.7 (±9.52)^{ab}	267^{ab}
23-3	10.5^{c}	8.6 (±1.29)^{ab}	72^{ab}	40.7 (±13.71)^{ab}	307^{ab}
WT*	0.0^{d}	11.0 (±2.32)^{a}	120^{a}	67.0 (±12.46)^{a}	570^{a}

* Wild type plant (negative control); Nt, Not tested. Different letters in the same column represent significant differences by Duncan's multiple range test (P = 0.05).

Table 2. Population growth and mortality of cotton aphids (*Aphis gossypii*) in non-choice leaf disk bioassays of chrysanthemum 1581 transformed with the SAE gene.

Lines	SAE gene expression	No choice leaf disk bioassay (5 nymphs, 5 days, n = 5)		
	qRT-PCR (%)	Mean of population (total)	Mean of dead aphids	Mean % mortality (dead/total per replicate)
15-4	100^{a}	15.6 (±3.7)^{a}	4.4 (±0.51)^{a}	32 (±6.3)^{a}
31-1	71.0^{a}	17.0 (±4.5)^{a}	2.8 (±0.37)^{bc}	27 (±13)^{ab}
27-2	33.7^{b}	17.8 (±3.7)^{a}	2.6 (±0.68)^{bc}	18 (±5.0)^{bc}
18-4	33.7^{b}	18.4 (±1.5)^{a}	2.8 (±0.2)^{bc}	16 (±2.1)^{bc}
8-2	11.2^{c}	14.6 (±1.2)^{a}	3.0 (±0.55)^{abc}	22 (±5.0)^{abc}
23-3	10.5^{c}	13.8 (±1.6)^{a}	3.4 (±0.4)^{ab}	26 (±4.0)^{ab}
WT*	0.0^{d}	17.6 (±3.0)^{a}	1.6 (±0.51)^{c}	11 (±3.0)^{c}

* Wild type plant (negative control); Nt, Not tested; A. U. arbitrary unit. Different letters in the same column represent significant differences by Duncan's multiple range test (P = 0.05). % Mortality is calculated per replicate (n = 5) and the average is given.

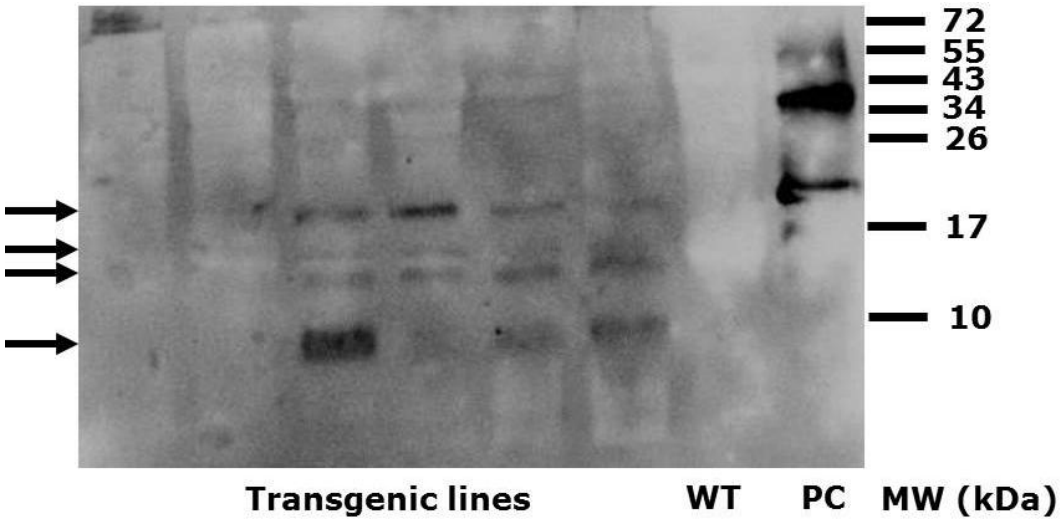

Figure 5. Western-blot analysis of the equistatin protein accumulated in chrysanthemum plants. PC: Positive control protein, produced in *E*. *coli*. WT: wild type plant (negative control), M: size marker.

PCR (Table 1). Also, in whole plant bioassays, transgenic lines 15-4 and 31-1 with the highest expression levels had 58 to 69% lower aphid population (Table 1). Cotton aphids were tested only in a non-choice bioassay on leaf discs. Similar to the artificial diet bioassays, these insects responded much more sensitively to the SAE protein product. The mortality increased significantly from 11% in control lines to 27 to 32% on transgenic lines 15-4 and 31-1 (Table 2).

DISCUSSION

Aphids are worldwide important pests and virus vectors. The search for a chemical basis of genetic plant resistance against aphids has resulted in a range of compounds and proteins with antibiotic and/or repellent/ deterrent activities. These mainly feature various lectins, serine and cysteine protease inhibitors, acyl sugar esters and some mono- and sesquiterpenes (Wu et al., 2006; Rahbé et al., 1995, 2003ab; Stoger et al., 1999; Nagadhara et al., 2003; Yao et al., 2003; Gatehouse et al., 1999; Goffreda et al., 1990; Aharoni et al., 2003). In the present study, we demonstrate that the cysteine and aspartic protease inhibitor sea anemone equistatin (SAE) is a highly potent insecticidal protein against a range of different aphids that are commonly found on chrysanthemum. The pea aphid and cotton aphid have LC_{50}s of 190 to 203 µg ml^{-1}, while the peach aphid was suffering from significant growth reductions on artificial diets containing SAE protein in that concentration range. Previously, SAE has been expressed in potato at a maximum expression level of 3 to 7% of total protein, when the SAE protein was carrying a KDEL signal at its C-terminus (Outchkourov et al., 2004a). If we can assume that plant leaves contain 15 mg gFW^{-1} of protein, then an expression level of 1% roughly represents 150 µg ml^{-1}, which is in the range of the required LC50 concentrations for pea and cotton aphid. Earlier experiments with Arabidopsis had shown that when SAE carried a KDEL signal; however, that resistance to aphids was not observed, presumably as a result of different targeting, leading to differential exposure to the protein (results not shown). Experiments with chrysanthemum were, therefore, limited to SAE without KDEL tag, which presumably secretes SAE by default into the apoplast. In potato that construct yielded an average expression level of 0.36% of total protein based on the strong RbcS1 rubisco small subunit promoter from chrysanthemum (Outchkourov et al., 2002), but the strong degradation of SAE in transgenic chrysanthemum as seen in the western blots did not allow us to make similar assessments of the actual protein expression level.

Alternative to protein blots expression levels could also be ranked on the basis of mRNA levels, and in this way correlations could be established between the expression levels and the growth reduction of peach aphid and mortality of cotton aphid. The growth reductions on plants for peach aphid were much higher than observed on artificial diet. The reasons for this are difficult to answer, but might be related to the fact that artificial diets are protein free, only containing free amino acids. This may create a lesser dependence on proteolytic activity on those diets compared to plant phloem sap. As reported for potato by Outchkourov et al. (2003b), also in this study, two thirds of the SAE protein appeared to be processed into several distinct bands in the early morning, and in the late afternoon, no detectable signal remained (data not shown). It is not known for chrysanthemum how

the first steps of this degradation affect the bioactivity of the SAE. In principle, the separate domains may still be active as was demonstrated in potato by affinity purification of the degradation products using a papain column. The distinct bands are target for further proteolysis; however, as afternoon and night levels were no longer detectable. Potential ways of preventing this degradation are co-expression of additional cysteine protease inhibitors which target the plant proteases. That method was shown to work well for potato and may work for chrysanthemum as well (Outchkourov et al., 2004b).

The SAE gene is potentially useful for the genetic engineering of enhanced aphid and thrips resistance in chrysanthemum and other crops. Chrysanthemum is mostly grown in greenhouses, which makes our tests on whole plants representative of commercial conditions. Nevertheless, tests were done on small plants only and in closed containers, so that a long term study on the effects of this gene on the population build up on these plants remains to be done. Furthermore, aphids tend to accumulate most on plants that are forming flower buds. Analysis of these stages will therefore need to be done in a follow up study as well. In that case, it will also be relevant to study whether SAE changes probing behaviour and thus could affect the transmission of viruses.

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African Journal of Biotechnology

Full Length Research Paper

Adsorption of violet B by agricultural waste of soft pistachio shells

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In this study, inexpensive agricultural waste pistachio sells was used for adsorption of violet B. Fourier transform infrared spectroscopy (FTIR) and scanning electron microscope (SEM) were used to characterize the pistachio shells. The morphology of pistachio shell was studied by SEM and it showed the porous structure of pistachio shells. Different parameters like contact time, pH and mass of sorbent were investigated. The adsorption is dependent on pH of the medium where the removal efficiency increases as the pH turns to 11. The contact time studies showed that 45 min shaking time was sufficient to achieve the equilibrium. The reaction kinetics data were analyzed using two reactions kinetic of pseudo-first-order reaction model and pseudo-second-order reaction model, and it was found that the removal of violet B followed the pseudo-second order reaction model.

Key words: Adsorption, violet B, agricultural waste, pistachio shell.

INTRODUCTION

Dyes are widely used in the textile industry to color products. One of the major problems concerning textile wastewaters is colored effluent. This wastewater contains a variety of organic compounds and toxic substances, which are harmful to fish and other aquatic organisms (Wang et al., 2008). Many physical and chemical methods such as coagulation, precipitation and oxidation have been used for treatment of dye containing effluent (Annadurai et al., 2002; Nigam et al., 1996). Adsorption has gained favor due to proven efficiency in the removal of pollutants from effluents to stable forms for the aforementioned conventional method.

Adsorption using activated carbon is an effective purification and separation technique used in industry especially in water and wastewater treatments that can remove heavy metals and organic compound from wastewater. Activated carbon surfaces have a pore size that determine its adsorption capacity, a chemical structure that influences its interaction with polar and non-polar adsorbates, and active sites which determine the type of chemical reactions with other molecules (Velmurugan et al., 2011; Sundaram and Sivakumar, 2012; Hashemian and Salimi, 2012). Several kinds of sorbents were examined for removal of dyes from water (Zhang et al., 2007; Wu et al., 2005, 2004; Wu and Qu, 2005; McKay, 1983; Ramakrishna and Viraraghavan, 1997; Namasivayam and Yamuna, 1995). Different kinds of activated carbon have been achieved from different agriculture wastes and used as low-cost adsorbents for removal of organic compounds and dyes from aqueous solution (Mohan et al., 2005; Bhatti et al., 2008; Elnemr et al., 2009; Liang et al., 2009; Annadurai et al., 2002; Hashemian, 2011; Hashemian and Mirshamsi, 2012; Robinson et al., 2002). Carbon produced from nutshells of walnut, hazelnut, pistachio, almond, and apricot stone were prepared. Adsorption of copper, zinc, lead and cadmium that exist in industrial wastewater has been studied (Kazemipour et al., 2008).

Attia who investigated the two activated carbons were obtained from pistachio shells by impregnation with

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Table 1. The properties of pistachio shells.

Parameter	Value
Carbon (%)	51.2
Oxygen (%)	42.5
Hydrogen (%)	5.3
Ash content (%)	0.8
Moisture content (%)	4.92
Surface area (m^2 g^{-1})	17.8

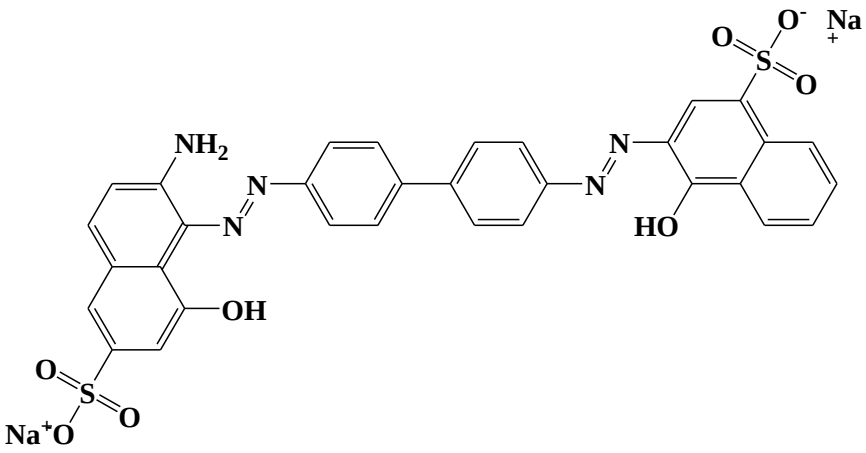

Figure 1. Chemical structure of violet B.

H_3PO_4 and heat treatment. Adsorption isotherms of methylene blue, rhodamine B, phenol and *p*-nitrophenol were determined at room temperature, from aqueous solutions (Attia et al., 2003). Pistachio shells are inexpensive and environmentally friendly sorbent. The major objective of this work is possibility of using cellulose-based waste soft pistachio shells for adsorption of violet B from water.

MATERIALS AND METHODS

Soft pistachio shells were used as starting materials. Pistachio shells were collected from a local center of preparation of shell-removed pistachio (IRAN- Yazd). They were washed thoroughly with double-distilled water to remove adhering dirt. The pistachio shells were preheated in an oven at 100°C for about 48 h to reduce the moisture content. They were then crushed with a high speed mill and sieved on a sieve mechanical shaker, and the size fraction of lower than 180 µm that has been passed through US standard sieve number 80, was used in this study. Some of the properties of pistachio shells are summarized in Table 1. All chemicals were of analytical reagent grade. Distilled water was used for all of the experiments. 1-Naphthalenesulfonic acid, 3-((4'-((2-amino-8-hydroxy-6-sulfo-1-naphthalenyl)azo)(1,1'-biphenyl)-4-yl)azo)-4-hydroxy-, disodium salt (violet B) was purchased from Merck. Violet B is double azo class with molecular formula of $C_{32}H_{21}N_5Na_2O_8S_2$ (MW = 713.65). It was used as received without further purification.

The percent removal of violet B by the hereby adsorbent is given by:

$$\% \text{ removal} = (C_0\text{-}C_e)/C_0 \times 100$$

Where, C_0 and C_e denote the initial and equilibrium dye concentration (mg L^{-1}).

The stock solution of 1000 mg L^{-1} of VB dye was prepared in double distilled water. The dilution was done in distilled water when necessary. Chemical structure of violet B is shown in Figure 1. IR measurements were performed by FTIR tensor-27 of Burker Co. Pressed pellets were prepared by grinding the powder specimens with spectroscopic grade KBr for FTIR spectra test. UV-Vis spectrophotometer 160 A Shimadzu was used for determination of concentration of dye. Scanning electron microscopy was performed using a Philips SEM model XL30 electron microscope. The pH was adjusted by addition of HCl or NaOH. All pH measurements were carried out with an ISTEK- 720P pH meter. The suspensions containing 0.2 g of the sorbent with varying amounts of dye was shaken in an orbital shaker at 150 rpm.

RESULTS AND DISCUSSION

Characterization of adsorbent

FTIR spectrum of pistachio shell is shown in Figure 2. The broad peak around 3410 cm^{-1} correspond to O-H

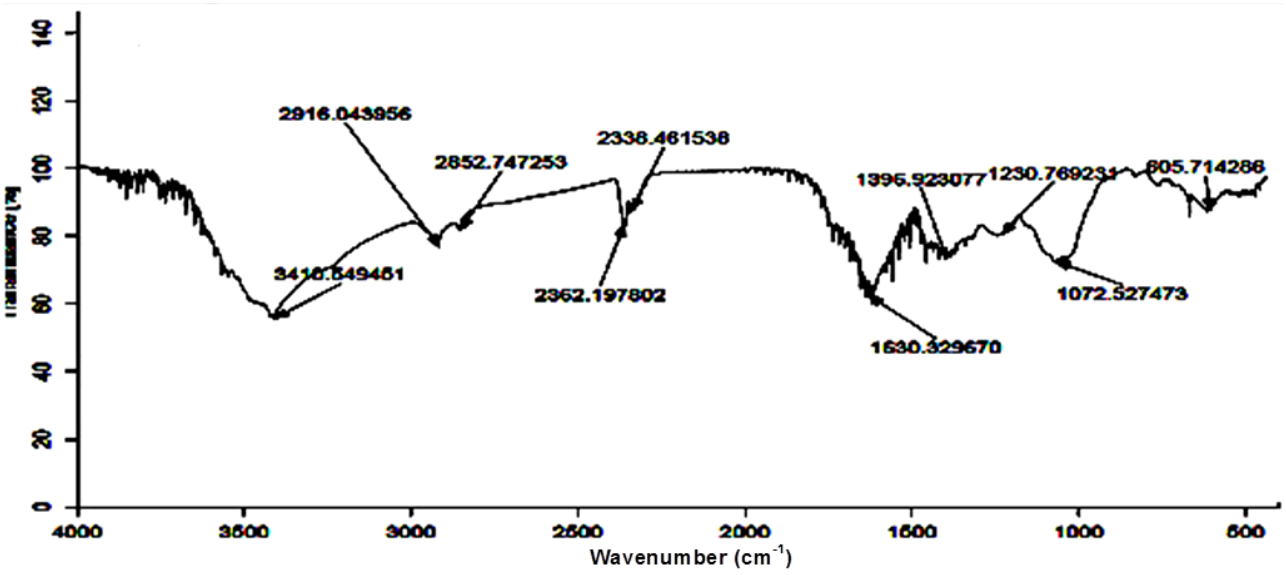

Figure 2. FTIR spectra of pistachio shell.

stretching vibrations, due to inter and inter-molecular hydrogen banding of polymeric compounds (macromolecular associations) such as alcohols, phenols and carboxylic acids, as in pectin, cellulose groups on the adsorbent surface. The peaks at 2916 and 2852 cm^{-1} are attributed to the symmetric and asymmetric C-H stretching vibration of aliphatic acids. The peaks around 1395 cm^{-1} are due to the symmetric bending of CH_3. The peak observed at 1630 cm^{-1} is the stretching vibration of bond due to non-ionic carboxyl groups (-COOH, -$COOCH_3$), and may be assigned to carboxylic acids or their esters. Broad peak at 1072 cm^{-1} may be due to stretching vibration of C-OH of alcoholic groups and carboxylic acids (Mehrasbi et al., 2009).

The morphology of pistachio shells were studied by SEM. Figure 3 shows the view of pistachio shell. It also shows the agglomeration of many micro fine particles, which led to a rough surface and the presence of pores structure. This is suitable for sorption and increases the sorbent capability to remove dye.

Effect of contact time

The effect of contact time on the adsorption of violet B onto pistachio shells is shown in Figure 4. Contact time is an essential parameter in all transfer phenomena such as adsorption. 30 ml of 15, 25 and 35 mg L^{-1} of violet B and 0.2 g of pistachio shells was used. The results show the adsorption increases with increase of contact time. Obviously, the adsorption equilibrium was attained after stirring for 45 min. It was found that at last more than 70% of adsorption process of violet B occurred in the first 45 min, and thereafter the rate of adsorption was found to be slow. The rapid adsorption at the initial contact time is due to the availability of the porosity surface of sorbent which led to fast adsorption of dye molecules. The UV spectra of violet B and violet B loaded pistachio shell was studied. The spectrum is show in Figure 5. These absorbance peaks decreased in intensity as the treatment time increased, and after treatment for 60 min, this peak almost disappeared, which indicates the violet B diminish after adsorption and adsorbed onto surface of pistachio shell.

Effect of pH

Figure 6 shows the effect of pH on the adsorption of violet B onto pistachio shell. The adsorption capacity increased with increasing pH of the solution. The maximum adsorption capacity of violet B on pistachio shell was observed at pH 11. This could be explained by the fact that at low pH, more protons will be available to protonate hydroxyl groups, reducing the number of binding sites for the adsorption of violet B. Violet B is cationic dye, which exist in aqueous solution in the form of positively charged ions. The point of zero charge of pistachio shells was at pH 6.5 to 7.5.

Therefore, at low pH (below the point of zero charge), the exchange sites on the pistachio shell become positive, the violet B dye compete with the H^+ ions in the solution for the active sites and consequently lower adsorption happened. At high pH values (above point of zero charge), surface of the pistachio shells had a higher negative charge which results in higher attraction of violet B. Hence, adsorption of violet B onto pistachio shell is optimum in the pH range of 10 to 11. At pH 11 a signifi-

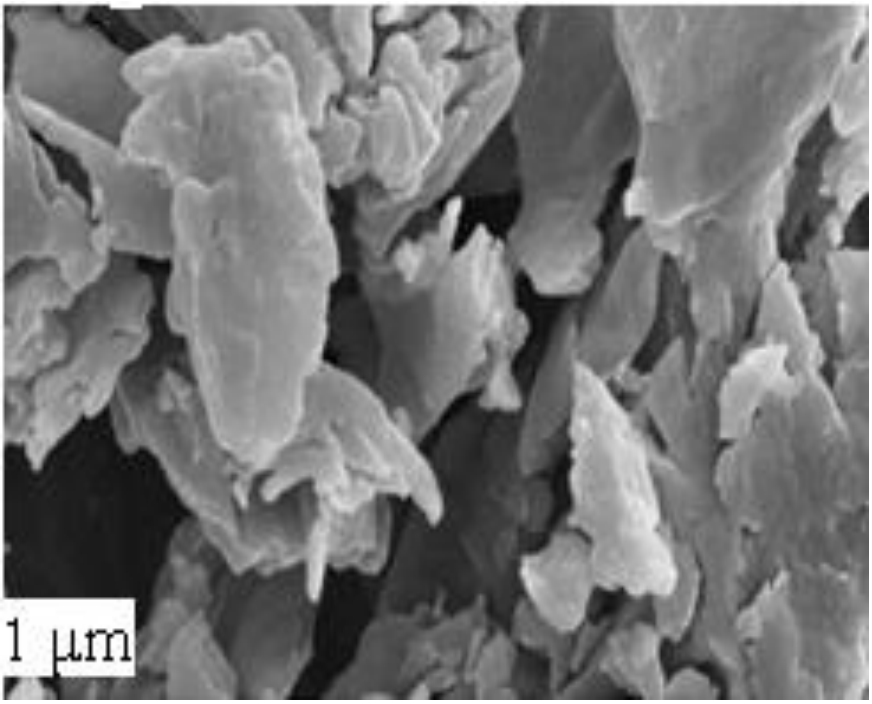

Figure 3. SEM image of pistachio shells.

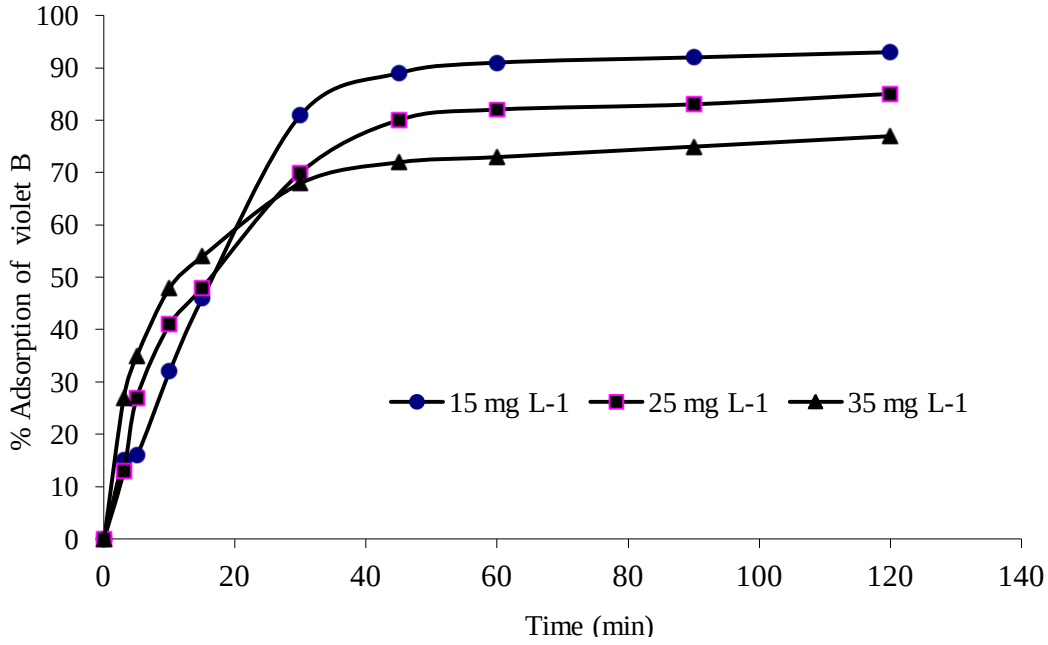

Figure 4. Effect of contact time for adsorption of violet B onto pistachio shell.

cantly high-electrostatic attraction exists between the negatively charged surface of the adsorbent and dye. As the pH of the system decrease, the number of positively charged sites increased and the number of negatively charged sites decreased. A positively charged surface site on the adsorbent does not favor for adsorption of violet B dye due to the electrostatic repulsion. The adsorption behavior showed that adsorption of violet B onto pistachio shell is governed by electrostatic interactions.

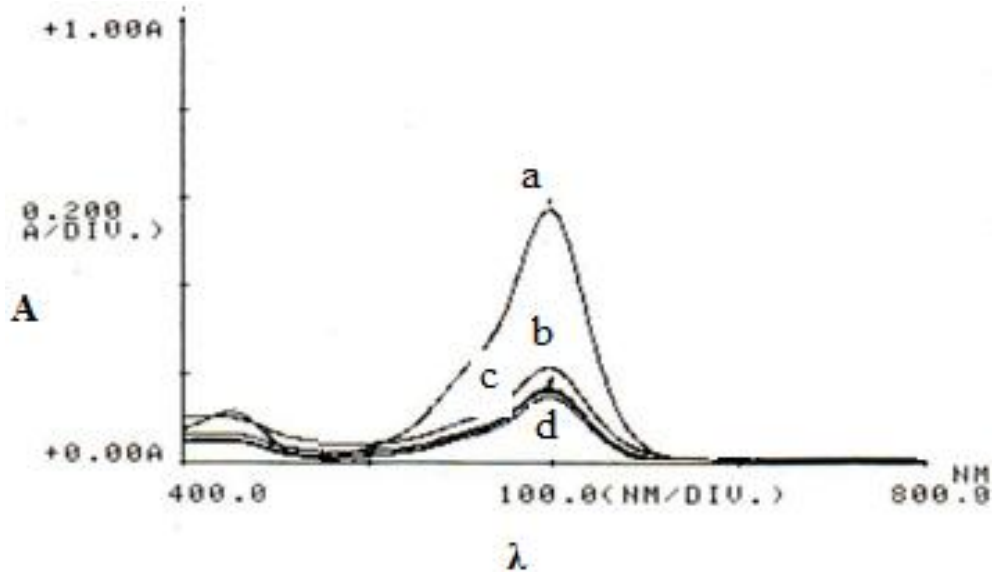

Figure 5. UV-vis electronic spectra of violet B before and after adsorption at different time: a-violet B, b-after 15 min, c-after 30 min, d-after 60 min of contact time with pistachio shell.

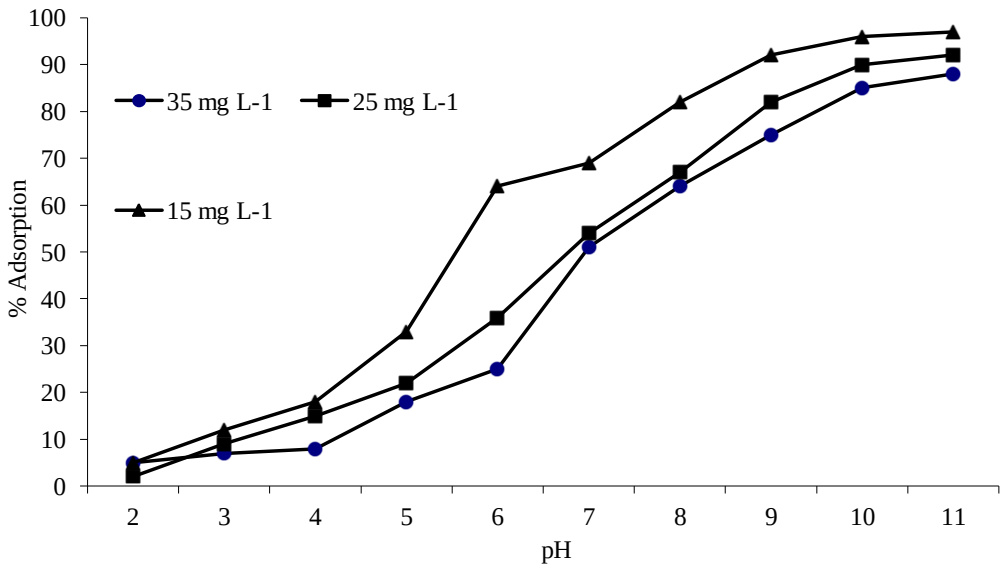

Figure 6. Effect of pH for adsorption of violet B onto 0.2 g pistachio shell for 30 ml violet B.

Effect of adsorbent mass on dye adsorption

The adsorption of violet B onto pistachio shell was studied by varying the quantity of adsorbent (0.1 to 3.0 g /30 ml) in the test solution at initial violet B concentration (25 mg L^{-1}), temperature (25±2°C) and pH 11 at contact time for 45 min. The percent of adsorption was increased when adsorbent dose increased. The adsorption increased rapidly, when pistachio shell dosage was increased from 0.1 to 1 g/30 ml at equilibrium time of 45 min. Increase in the adsorption with adsorbent quantity can be attributed to increased pistachio shell surface area and availability of more adsorption sites (Hashemian and Salimi, 2012).

Adsorption kinetics

The reaction kinetics data were analyzed using two reaction kinetic; pseudo-first-order reaction model and pseudo-second-order reaction model. The adsorption kinetic data were described by the pseudo-first-order

Table 2. Kinetic parameters for the removal of violet B by pistachio shell

Sample (mg L^{-1})	First order		Second order	
	R^2	K_1 (s^{-1})	R^2	K_2 (g mg^{-1} min^{-1})
VB (15)	0.850	1.72×10^{-5}	0.994	2.35×10^{-4}
VB (25)	0.866	1.86×10^{-5}	0.996	1.965×10^{-4}
VB (35)	0.750	1.94×10^{-5}	0.982	1.265×10^{-4}

model (Lagergren, 1898), which is the earliest known equation describing the adsorption rate based on the adsorption capacity. The equation of pseudo-first-order model is commonly express as follows:

$$\ln (q_e - q_t) = \ln(q_e) - k_1 t$$

Where, q_e and q_t are the amounts of dye adsorbed per unite mass of the adsorbent (mg g^{-1}) at equilibrium and time t, respectively; and k_1 is the rate constant of adsorption (min^{-1}). When ln (q_e - q_t) was plotted against time, a straight line should be obtained with a slope of k_1, if the first order kinetics is valid.

The adsorption kinetic may be described by the pseudo-second order model (Ho et al., 2000), which is generally given as follows:

$$t/ q_t = 1(k_2 q_e^2) + t / q_e$$

Where, q_e and q_t represent the amount of dye adsorbed (mg g^{-1}) at equilibrium and at any time and k_2 is the rate constant of the pseudo-second order equation (g mg^{-1} min^{-1}). A plot of t/q versus time (t) would yield a line with a slope of 1/ q_e and an intercept of 1/ ($k_2 q_e^2$), if the second order model is a suitable expression.

It was mentioned that the curve fitting plots of ln (q_e − q_t) versus t does not show good results for the entire sorption period, while the plots of t/q_t versus t give a straight line confirming the applicability of the pseudo-second-order equation. Values of k_2 and equilibrium adsorption capacity q_e were calculated from the intercept and slope of the plots of t/q_t versus *t*, respectively. The values of R^2 and q_e also indicate that this equation produced better results (Table 1). R^2 values for pseudo-second-order kinetic model were found to be higher and the calculated q_e values were mainly equal to the experimental data. This indicates that the adsorption process follows the pseudo-second-order kinetic model for the entire sorption period. The pseudo-first-order and pseudo-second-order models of adsorption for 30 ml of 25 mg L^{-1} of violet B at room temperature were investigated (Table 2).

Conclusions

The pistachio shell as low-cost agricultural waste material was prepared as adsorbent for adsorption of azo dyes from aqueous solutions. The adsorption process was attained equilibrium within 45 min of contact time and pH 11. Kinetic studies were made for the adsorption of violet B from aqueous solutions onto pistachio shell. The kinetic was found to be best-fit pseudo-second- order equation.

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Full Length Research Paper

Determination of heavy metals and genotoxicity of water from an artesian well in the city of Vazante-MG, Brazil

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The city of Vazante-MG is of great socioeconomic and environmental interest because it is the most important zinc producer district of Brazil. The mineral processing and geochemical processes may determine high concentrations of heavy metals in water intended for human consumption. Thus, the present study aimed to quantify and evaluate the heavy metal genotoxicity of artesian water in the city by Atomic absorption spectrophotometer analysis and testing with the *Allium cepa* test, respectively. This study reveals a chemical contamination in well water in the city, caused by the presence of heavy metals. Therefore, it can be considered that the high levels of heavy metals found in water samples are correlated with the genotoxic events observed in root cells of *A. cepa*.

Key words: *Allium cepa*, micronucleus, atomic absorption, chromosome aberration, mitotic index.

INTRODUCTION

The Vazante-MG region is of great socioeconomic and environmental interest, since it is the most important zinc producer district of Brazil (Hitzman et al., 2003). The inability of differentiating geogenic anomalies from those that result from processes of contamination related to human studies, suggests the need of assays to evaluate the presence of heavy metals in soil and water in order to contribute to a better evaluation of the occurrence of contamination by these metals (Borges Júnior et al., 2008). Preliminary evaluation of a suspected area of contamination is performed based on information available (CETESB, 1999, 2001). The area is considered contaminated if the concentration of elements or substances of interest are above the given threshold, which indicates the potential deleterious effect on human and animal health (Junior Borges et al., 2008a).

The effects of mineral processing together with the geochemical processes that naturally occur in reason of the soil characteristics and the entrainment of heavy particles to the aquatic system may provide high concentrations of heavy metals in water intended for domestic consumption (Yabe et al., 1998;, Guedes et al., 2005). The presence of heavy metals in groundwater may occur due to contact with rivers and lakes contaminated with sewage or by leaching by precipitation of contaminated soils (Di Natale et al., 2008).

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Abbreviations: MMS, methylmetanosulfonate; **MI,** mitotic index; **CA,** chromosome aberrations; **MN,** micronucleus.

Heavy metals are among the most common inorganic pollutants in water (Chandra et al., 2005). They are highly distributed over the earth's crust (Arambasic et al., 1995; Min et al., 2013) and represent one of the most toxic environmental pollutants (Ghosh, 2005; Sharma, 2009). Intoxication with heavy metals has been observed in many parts of the world, usually related to chronic exposure in environment through contamination of drinking water (Hang et al., 2009; Singh and Kalamdhad, 2013). Epidemiological evidence has shown that a long-term exposure is highly associated with increased risk of development of several diseases, including cancers (Zhuang et al., 2009). *In vivo* and *in vitro* assays have shown that heavy metals induce chromosomal aberrations and micronucleus in plant and animal (Rank et al., 1998; Majer et al., 2002; Rodriguez-Cea et al., 2003). Therefore it became important to carry out the environmental monitoring of water intended for human consumption.

Only with the chemical analyzes of water, it is not possible to determine the ecotoxicological risk that chemicals present in it can cause to the bodies, since such analysis alone does not indicate toxicity (Fuentes et al., 2006). Therefore, ecotoxicity tests among them those of environmental mutagenesis, have been proposed and applied to understand the genetic and physiological responses of exposed organisms (White et al., 2004; Chen et al., 2004). Bioassays with plants, such as the *Allium cepa* test, have some advantages over the tests in mammalian cells and microorganisms for environmental monitoring (Grant, 1994; Radic et al., 2014; Osakca & Silah, 2012). Plant assays are highly sensitive to many environmental pollutants, including heavy metals (Fiskesjo, 1985; Smaka-Kincl et al., 1996; Steinkellner et al., 1998; Fatima et al., 2005; Yi et al., 2007; Egito et al., 2007; Pesnya and Romanovsky, 2013). The use of the *A. cepa* bioassay is suggested because it is known that many plants are damaged by heavy metal contamination (Minissi et al., 1997; Amaral et al., 2007; Smith, 2001). This study aimed to quantify and evaluate the heavy metal genotoxicity of artesian water in the city of Vazante-MG/Brazil by atomic absorption spectrophotometer analysis and testing of the *A. cepa,* respectively.

MATERIALS AND METHODS

Samples collection

Water samples were collected at Vazante - MG/Brazil (S 17º 59'27 "W and 46º 54'04", altitude, 638 m) in May 2007, directly in wells registered by the Companhia de Saneamento de Minas Gerais (COPASA). Two collection points were determined: Sample 1 (S1): Water from the borehole, without treatment; Sample 2 (S2): water from an artesian well with simplified treatment (disinfection and fluoridation) in accordance with the rules of COPASA.

Three samples of 5 liters were collected from each point considered, being collected at intervals of 10 min and stored in sterile flasks, totaling 15 l per point. The pH of the samples was measured in the field, using a manual pH meter (Lutron pH-208).

The samples were transported in an isothermal box to the Laboratory of Chemistry and Instrumental Analytical Center of the University Center of Patos de Minas - UNIPAM.

Determination of heavy metals

For the heavy metals analyses, from each sample 100 mL was taken, 20 mL of nitric acid PA was added and then heated to evaporate until it remained 60 mL solution. After reaching room temperature, 40 mL of ultrapure water was added to obtain a final solution of 100 ml of sample for testing. The reading of the heavy metals in water was performed in triplicate and measured by the atomic absorption spectrophotometer Perkin Elmer 3300. In the present study, we analyzed the following metals: Cadmium, lead, copper and zinc. The gases used for reading were acetylene and compressed air in the flame analysis with hollow cathode lamp, a procedure performed in accordance with Santos et al. (2006).

Allium cepa test

Treatment

The experiment was conducted at the Laboratory of Plant Physiology, Centro Universitário de Patos de Minas, Minas Gerais and analyzed at the Laboratory of Herbal Medicines, Universidade Estadual Paulista, Assis-SP/ Brazil. The experimental protocol was essentially performed as described by Ma et al. (1995). Twelve (12) bulbs were exposed to water samples collected, six for samples collected in S1 and six for those collected in S2. Twelve (12) other bulbs were destined to negative control groups (NC) and positive control (PC), which were prepared using mineral water and methylmetanosulfonate (MMS) to 10 mg / L (MMS, Sigma-Aldrich®, CAS 66-27 - 3), respectively.

Exposures were performed for a fixed period of 48 h for all treatments, except for the PC group that was exposed for 6 h according to the methodology described by Fiskesjö (1988) and Majer (2003). After the exposure period of the roots, they were fixed in acetic acid and ethanol solution (1:3) for 24 h. After fixation, the roots were transferred to a solution of 70% ethanol and kept in refrigerator at an average temperature of 4°C.

Determination of mitotic index, chromosome aberration and micronucleus

For preparation of the slides, roots were hydrolyzed in 1 N HCl at 60°C for 8 min and then stained with 2% solution of carmine in 45% acetic acid. The roots were then placed on a slide and the first millimeter removed from the apex of the root, so that the meristematic region corresponding to 2 mm and F1 cells were isolated for analysis by optical microscope. 1000 cells were counted per slide in an increase of 400 times, with 5 slides per treatment and control, and mitotic division stages, aberrant anaphases and telophases, and the frequency of micronucleus were quantified. The mitotic index was calculated according to the equation:

IM [%] = the number of dividing cells (1000 per slide) / number of cells analyzed x 100

For analysis of chromosomal aberrations (aberrant anaphases and telophases) and micronucleus frequency were performed according to methods previously described by Grant (1982) and in accordance with adjustments made by Yildiz et al. (2009).

Determining the length of the root

After the period of exposure and collection of the roots, the

Table 1. Results of MI, CA (anaphase and telophase) and MN in *Allium cepa* meristematic cells and root end length after treatment with water samples.

Sampling	Mitotic index	% Aberration chromosome		Micronucleated cells (%)	Length root (mm)
		Anaphase	Telophase		
NC	12.68±0.79	0.86±0.02	0.53±0.07	0.112±0.003	48.24±2.27
S1	5.11±0.13[ab]	11.47±0.52[ab]	9.11±0.16[ab]	3.374±0.123[ab]	19.31±2.09[ab]
S2	9.14±0.57[ab]	7.94±0.37[a]	5.36±0.37[a]	2.658±0.017[a]	23.17±1,93[ab]
PC	13.46±1.17	5.06±0.46	4.23±0.16	1.680±0.038	33.42±2.46

5000 cells analyzed per treatment. Mean±S.D. [a], Significantly different from negative control ($p < 0.05$), according to Kruskal–Wallis test. [b], significantly different from positive control ($p < 0.05$), according to Kruskal–Wallis test.

measurement of the length in millimeter of the roots was performed with the help of a digital caliper (DIGIMESS®), having a total of 25 roots per treatment.

Statistical analysis

The mitotic index, frequencies of chromosomal aberrations and micronucleus obtained for each treatment during the period between exposure and the samples were compared with the controls and analyzed statistically using the Kruskal-Wallis test ($p < 0.05$), as described by Grisolia et al. (2005) and Rudder et al. (2008).

RESULTS AND DISCUSSION

The pH of samples S1 and S2 varied from a minimum of 6.55 (S1) to a maximum of 6.65 (S2), with an average of 6.60. In its resolution of CONAMA (2005), permitted range is 6.5 to 7.5, so all values remained in that range (Guedes et al., 2005).

Figure 1 shows the concentrations of cadmium, copper, lead and zinc found in the different water samples (S1 and S2) and the maximum tolerable in the environment of each metal recommended by the WHO (1998) and according to CONAMA (2005). Both samples showed high levels of all analyzed metals which exceed the maxi-mum amount indicated and recommended by the rele-vant authorities. The sample S2 showed lower values when compared to sample S1, but all metals exceeded the maximum tolerated. The cadmium concentration in the sample S2 was 0.045 mg/L, or 4400% above recommended levels, the copper concentration was 0.086 mg/L, 855% above the indicated concentration, zinc showed 0.195 mg/L, exceeding 8% maximum tolerable concentration and lead showed 1341mg/L, being the metal with the highest value of the S2 sample, exceeding 13310% the recommended maximum. In relation to the S1 sample, it showed values for cadmium in excess of 4900% than the recommended maximum value, the value of copper exceeded 1044%, zinc exceeded 15% and lead exceeded 29410%, being the last one the highest of all metals analyzed according to the indicated maximum values (Figure 1).

According to Raskin and Ensley (2001) and Andrade et al. (2009) increased levels of heavy metals may be associated with destruction of vegetation cover in mining areas which exacerbates soil degradation, promoting water and wind erosion and leaching of contaminants into groundwater, leading to progressive degree of contamination in other areas. As reported by Rigobello et al. (1988) and Borges Júnior et al. (2008b) the region of Vazante has high levels of zinc and lead, being the largest zinc producer district of Brazil.

In recent decades, environmental contamination with heavy metals has risen dramatically. It is known that certain heavy metals can cause DNA damage and carcinogenic effects in animals and humans, and are probably, related to its mutagenic activity (Ernst, 2002; Arora et al., 2008; Megateli et al., 2009). According to Knasmüller et al. (2009), the standard tests used to detect heavy metals is currently problematic because several carcinogenic metals result in negative information on bacterial gene mutation assays and genotoxicity assays with mammalian cells, but tests performed in plant cells have become known for being a quick and useful test system in biomonitoring (Majer et al., 2005). The advantages of these tests include the similarity of the plants chromosomes organization with the human, its sensitivity to changes in environment (Grant, 1994) and the possibility of studying the effects on a wide range of environmental conditions. Repeated application of these tests to assess the genotoxic risks present in natural waters (rivers and lakes), wastewater and drinking water has affirmed its utility (Blagojevic et al., 2009).

Thus, this assay evaluated the genotoxic activity of different water samples by mean of the *A. cepa* test. Table 1 shows the results of the mitotic index (MI), chromosome aberrations (CA), micronucleus (MN) and root length of *A. cepa* after treatment with water samples. The sample S1 showed significant differences in relation to positive and negative controls in all parameters analyzed, and its average MI was the lowest (5.11) than controls, NC (12.68), PC (13.45) and the length of the roots of the sample (19.31 mm) also had decreased compared to controls: NC (48.24 mm), PC (33.42 mm). Since the percentage of MN and CA increased compared to controls, the frequency of MN of S1 was 3.374%, while the frequencies of the NC was 0.112 and 1.680% of the PC. The percentage of AC of S1 also increased, both for anaphase (11.47%) and telophase aberrant (9.11%),

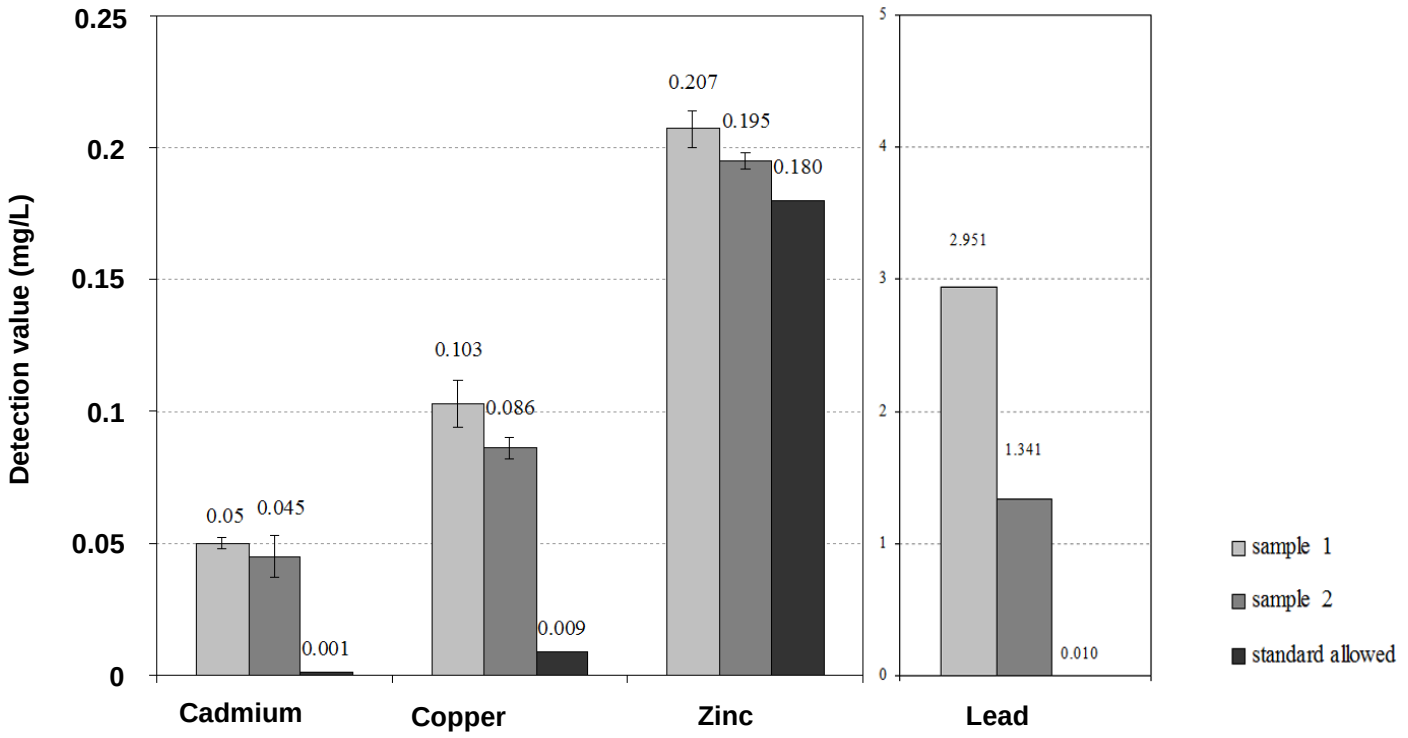

Figure 1. Concentration of heavy metals (cadmium, copper, lead and zinc) found in the water samples (S1 and S2) and maximum permitted under the Regulatory Determination CONAMA (2005) and recommended by WHO (1998).

while the negative and positive controls showed anaphase to 0.86 and 5.06% 0 and telophase, 53 and 4.23%, respectively.

The S2 differed from the positive and negative controls in relation to MI and length of roots. Both sample showed a decrease parameter, and the MI of the medium S2 is 9.14, while for the PC was 13.46 and the NC was 12.68. Values for the average length of the roots were 23.17 mm and S2 to the positive and negative controls were 33.42 and 48.24 mm, respectively. As for the parameters CA and MN percentage of the sample S2 showed no statistical difference in relation to the PC for both anaphase and telophase, but there were differences when compared to NC, and the percentages of both MN (2.658%) and AC (anaphase = 7.94% and telophase = 5.36%) increa-sed over the rate of MN, NC (0.112%) and the rate of CA of the same control (anaphase = 0.86% and telophase = 0.53%) (Table 1).

According to the results observed in this study, we consider that the high levels of heavy metals found in water samples are directly related to the genotoxic events observed in root cells of *A. cepa*. According to studies carried out by Seth et al. (2008), the high content of cadmium is associated with occurrence of chromosomal aberrations and increased frequency of micronuclei in the root of *A. cepa*. As shown by Glinska et al. (2007) and Ferraz et al. (2009), copper and its connections with macromolecules proved to be an effective cytotoxic agent and genotoxic in cell cultures and *in vivo* assays. Liu et al. (1994 and 2003) and Seregin et al. (2004) showed that the lead and cadmium inhibited root growth as a result of disruption of cell cycle and Wierzbicka (1988, 1989 and 1999), and Samardakiewicz Woz'ny (2005), Fusconi et al. (2006) showed a decrease in the mitotic cells of the root, where this value was accompanied by reduction in the number of cells in metaphase and anaphase. Furthermore, heavy metals, lead and cadmium induced c-mitosis, chromosomal adhesion and bridges, and besides that, lead also caused chromosome delay, nucleus with more condensed chromatin and inhibited cytokinesis.

Conclusion

The present study reveals a chemical contamination in artesian well water in the city of Vazante-MG caused by the presence of heavy metals. The decrease in mitotic index, reducing the average length of roots and increased frequency of chromosomal aberrations and micronucleus in root meristematic cells of *A. cepa* exposed to treatment may be correlated with the presence of certain heavy metals determined in our assay, as well as the interaction with other classes of environmental contaminants, which are probably the agents that together induced the genotoxicity observed in this assay. Taken together, these results show the importance of evaluating the genotoxicity of water wells in areas with mineral richness, especially in areas near active mining sites.

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Full Length Research Paper

Production of L-glutaminase and its optimization from a novel marine isolate *Vibrio azureus* JK-79

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L-Glutaminase is an amidohydrolase which is produced by a variety of micro-organisms including bacteria, yeast and fungi. It is currently used in the treatment of acute lymphoblastic leukemia (ALL) and human immunodeficiency virus (HIV). Another potential application of L-glutaminase is as flavor enhancing agent in soy sauce fermentation. Even though L-glutaminase activity was reported in numerous micro-organisms, production of L-glutaminase from marine bacteria is very scanty. With this view, in the present research work, L-glutaminase production pattern was studied under submerged fermentation using novel marine isolate *Vibrio azureus* strain JK-79 (GenBank Acession Number JQ820323) based on one-factor-at-a-time approach. The maximum yield of enzyme production (247 U/ml) was achieved in a seawater based medium at pH 8, 37°C, 1% inoculum concentration and 2% glutamine concentration for 24 h. The medium when supplemented with carbon source, it improved the enzyme production from 247 to 321 U/ml with 1.5% maltose. Addition of 2% soybean meal also improved the L-glutaminase production (289 U/ml). The above results indicate the scope for production of salt tolerant L-glutaminase using this novel marine bacterial strain.

Key words: L-Glutaminase, *Vibrio azureus* JK-79, submerged fermentation, optimization of fermentation, one-factor-at-a-time approach.

INTRODUCTION

L-Glutaminase (L-glutamine amidohydrolases E.C. 3.5.1.2) catalyses the hydrolysis of L-glutamine to glutamic acid and ammonia (Nandha et al., 2003). In recent years, it has gained much attention due to their potential application as anti-cancer agent (Roberts et al., 1970) and flavor enhancing agent (Yokotsuka, 1985).

Another most promising application of L-glutaminase is its usage in the treatment of human immunodeficiency virus (HIV) (Roberts et al., 1972). L-Glutaminase is also used in biosensors to monitor L-glutamine level in mammalian and hybridoma cell lines (Huang et al., 1995; Mulchandi et al., 1996). The tremendous application of L-glutaminase in various fields always prompted a search for a better source of the enzyme. Although L-glutaminase can be derived from both plant and animal sources, microbial source is generally preferred for industrial production due to their economic production, consistency, ease of process modification and optimization (Sabu, 2003).

Cancer cells, especially acute lymphoblastic leukemia (ALL) cells cannot synthesize L-glutamine and hence demand for large amount of L-glutamine for its growth. The use of amidases deprives the tumor cells from L-glutamine and causes selective death of L-glutamine dependent tumor cells. L-Glutaminase can bring about degradation of L-glutamine and thus can act as possible

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Abbreviations: HIV, Human immunodeficiency virus; **ALL,** acute lymphoblastic leukemia; **SWG,** sea water glutamine; **BSA,** bovine serum albumin.

Table 1. Composition of various fermentation media.

Medium No.	Medium	Composition of medium
1	Mineral Salts Glutamine medium (MSG) Renu and Chandrasekaran (1992)	L-glutamine-1%, D-Glucose-0.5%,Nacl-3%, KH_2PO_4 -0.1%, $MgSo_4.7H_2O$-0.05%, $CaCl_2$-0.01%, $NaNo_3$-0.01%, trisodium citrate- 0.01% and Distilled water-100ml , pH 6
2	Wakayama et al. (2005)	L-Glutamine - 2.0%, K_2HPO_4- 0.1%, KH_2PO_4- 0.1%, $MgSO_4$ $7H_2O$-0.01%, NaCl- 0.1% and Yeast extract -0.05%, Distilled water-100ml and pH 6.0
3	Sato et al. (1999)	D-Glucose -3.0%, yeast Extract- 0.5%, $MgSO_4$ -0.1% and KH_2PO_4 -0.1%, Distilled water-100ml and pH 6.0
4	Kumar and Chandrasekaran (2003)	L-Glutamine -1 %, D-glucose-0.05%, Distilled water-100 ml and pH 6.0
5	Sea water Glutamine (SWG) Kumar and Chandrasekaran (2003)	L-Glutamine -1 %, D-glucose-0.05%, aged sea water-100 ml and pH 6.0

candidate for enzyme therapy (Tanaka et al., 1988).

In recent years, L-glutaminase in combination with or as an alternative to L-asparginase could be used as in enzyme therapy for cancer particularly leukemia (Sabu et al., 2003). The marine biosphere is the richest habitat of microorganisms especially bacteria and also is the one which is less characterized. Hydrolytic enzymes from halotolerant micro-organisms provide an interesting alternative for therapeutic purposes as they are capable of functioning under conditions that leads to precipitation or denaturation of most proteins from terrestrial bacteria. Further, sea water, which is saline in nature and chemically closer to human blood plasma, could provide biomolecules especially enzymes that could have no or less side effects when used in therapeutic applications (Iyer and singhal, 2009).

L-glutaminase activity was reported in various terrestrial micro-organisms such as *Escherichia coli, Pseudomonas sp, Acinetobacter sp, Bacillus sp, Proteus morganni, Cryptococcus, Candida* and *Aspergillus oryzea* (Sabu, 2003). Apart from terrestrial sources, few marine micro-organisms were also known to synthesize L-glutaminase and include *Pseudomonas flurosence, Micrococcus luteus, Vibrio costicola* and *Beuveria bassiana* (Chandrasekaran, 1997).

In spite of its demonstrated potential as antileukemic agent, L-glutaminase is generally regarded as a key enzyme that controls the taste of fermented food such as soya sauce by increasing the glutamic acid content, there by imparting a unique flavor to the food (Nandakumar et al., 2003). Thus, salt tolerant and heat stable L-glutaminase demands not only search for potential strain, but also economically viable bioprocess for its large scale production (Nagendraprabhu G et al., 1995). From the literature, it is evident that only few reports are available on the extracellular production of L-glutaminase from marine bacteria and since there is an excessive requirement for salt and thermo tolerant L-glutaminases, a search for a potential marine strain that hyper produce this enzyme with novel properties and an economically viable bioprocess is pursued. Thus the present study, focuses on the L-glutaminase production from a potential and novel isolate *Vibrio azureus* JK-79 (JQ820323) under submerged fermentation and optimization of the process parameters and nutritional factors of fermentation for enhanced enzyme production.

MATERIALS AND METHODS

Micro-organism and culture maintenance conditions

The *V. azureus* JK-79 (JQ820323) used in this study was isolated from marine sediment collected from Parangipettai costal area (Lat. 11°.29' N; Long. 79°.46'E) (Kiruthika and Saraswathy, 2013). The culture was maintained in Zobell's marine agar slant (Himedia, India) at 4°C and was periodically sub-cultured.

Inoculum preparation

A loopful of culture from 24 h old Zobell's marine agar slant was inoculated on 10 ml of inoculum medium containing (g/l of aged sea water (30 ppt of salinity)): Peptone, 5; yeast extract, 1; NaCl, 2.45; and L-glutamine, 1. The inoculated medium was incubated at 37°C on rotary shaker at 120 rpm for 24 h.

Production media

Enzyme production was carried out in 500 ml Erlenmayor flasks. Five different types of production media (Table 1) were evaluated for the production of L-glutaminase enzyme. All the five different media were inoculated with the prepared inoculums at 10% level and incubated in an incubator at 37°C, 120 rpm for 24 h. After incubation 10 ml of the culture was collected and harvested by centrifugation at 10,000 rpm for 20 min and the supernatant was used as sample for enzyme assay.

Enzyme assay

Glutaminase was assayed according to the method described in the study of Imada et al. (1973). An aliquot of 0.5 ml of the sample was mixed with 0.5 ml of 0.04 M L-glutamine solution in the presence of 0.5 ml of distilled water and 0.5 ml of phosphate buffer (0.1 M, pH 8). The mixture was incubated at 37°C for 30 min and the reaction was arrested by the addition of 0.5 ml of 1.5 M trichloro acetic acid.

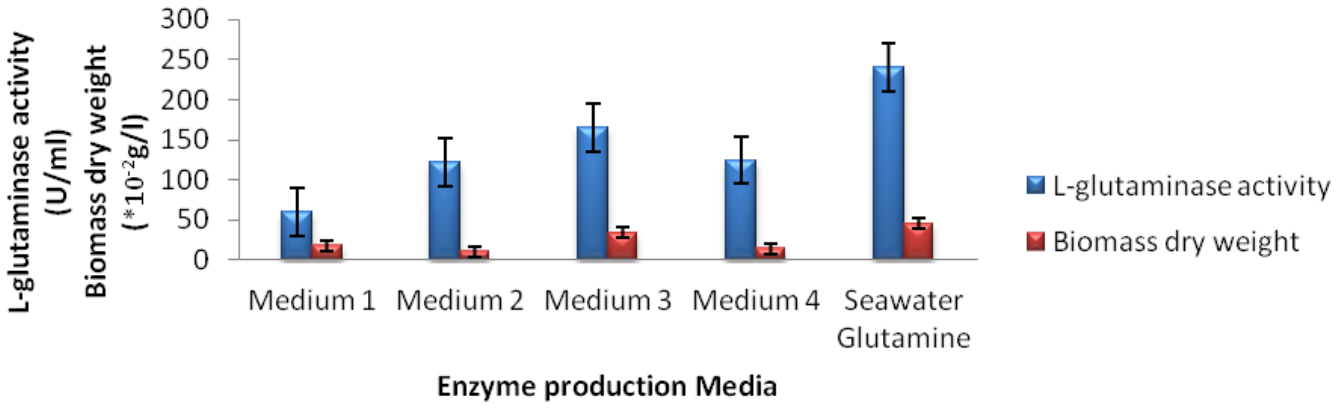

Figure 1. L-Glutaminase production from *Vibrio azureus* JK-79 and biomass concentration on different fermentation medium.

To 0.1 ml of the mixture, 3.7 ml of distilled water and 0.2 ml of Nessler's reagent were added. The absorbance was measured at 450 nm using a UV-visible spectrophotometer (Spectronic-Genesys5).

The liberated ammonia content was measured and one international unit of L-glutaminase was defined as the amount of enzyme that liberates one µmole of ammonia under optimal conditions. Assays were done in triplicates and the mean enzyme activity was expressed as units/ml of culture supernatant (U/ml).

Optimization of process parameters for L-glutaminase production

The sea water glutamine (SWG) medium was used as the basal medium and the process parameters were optimized one after another. After optimization of each parameter, it was included in the next study at its optimal level. The pH of the medium (5-9), incubation temperature (25-45°C), incubation time (24-120 h), inoculums concentration (1-5%) ,additional carbon source (D-fructose, sucrose, lactose, D-glucose, maltose and starch at 1% w/v), additional nitrogen sources (peptone, yeast extract, soybean meal, beef extract, meat extract, tryptone, ammonium sulphate, ammonium chloride, sodium nitrate and potassium nitrate at 1% w/v), and different amino acids (methionine, phenylalanine, histidine, cystein, L-aspargine, L-glutamic acid and L-glutamine at 1% w/v) were optimized for L-glutaminase yield. All experiments were conducted in triplicates and the mean values were alone taken into consideration.

Biomass estimation

Biomass was estimated in terms of dry weight and total cell protein. To determine the dry weight, the fermentation media was withdrawn at regular intervals of time and the turbidity was evaluated at 600 nm. From the absorbance, the dry weight was determined from the standard curve of absorbance vs. dry weight. Total cell protein was measured using the method of Herbert et al. (1971) which is essentially a modified Lowry's method and expressed as total cell protein (µg/ml). 0.5 ml of the sample was added to a test tube containing 0.5 ml of 1N NaOH. The tubes were closed with aluminium foil and the contents were boiled for 5 min in a water bath at 100°C. The mixture was cooled rapidly under tap water and 2.5 ml of reagent (0.5% sodium carbonate + 0.5% cupric sulphate solution in 1% sodium potassium tartarate). The tubes were kept for 10 min which was followed by addition of 0.5 ml of Folin's reagent. A blank containing 0.5 ml of distilled water instead of sample and a set of standard protein (bovine serum albumin) solutions were treated in the same way including the heating stage. After keeping for 30 min, allowing full colour development, the absorbance was measured at 750 nm in UV-visible spectrophotometer against the blank.

Enzyme protein

Protein content in the sample was estimated by Lowry's method (Lowry et al., 1951) using bovine serum albumin (BSA) as the standard and the values were expressed in (µg/ml).

RESULTS AND DISCUSSION

The microbial production of the enzyme depends on the genetic nature of the organism, the physio-chemical parameters, the fermentation medium components and their concentration. Hence, optimization of the above conditions is important to get maximal yields and to develop effective bioprocess system for industrial application. Many authors reported increased enzymes yield upon optimization of bioprocess conditions (Iyer and Singhal, 2008; 2009; 2010).

In the preliminary stage, a suitable fermentation medium or production medium was selected for the novel isolate *V. azureus* JK-79. Five different medium as given above were evaluated for the L-glutaminase production. Among the five media, maximal L-glutaminase production (240 U/ml), biomass (0.4561 g/l), enzyme protein (130 µg/ml) and total cell protein (170 µg/ml) were obtained from sea water glutamine (Figures 1 and 2) compared to other medium. Hence, it was preferred as optimum medium for the production of L-glutaminase.

Effect of pH on the medium

The pH of the fermentation medium is reported to influence the growth of any microbial strain and subse-

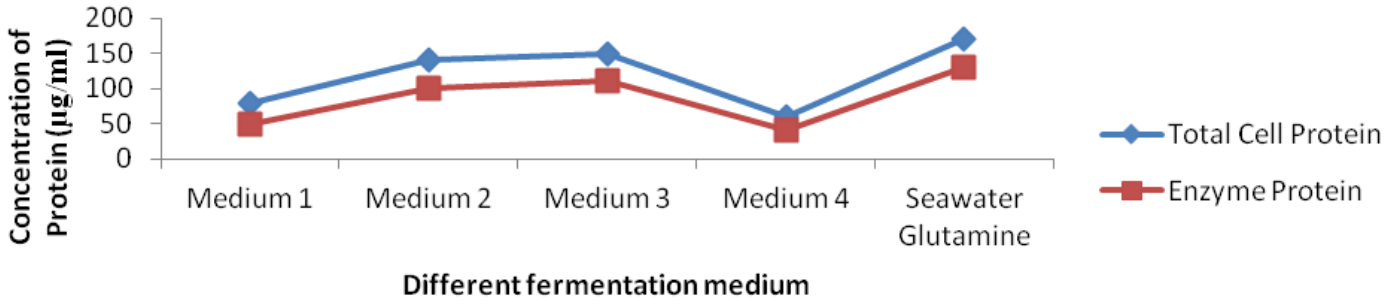

Figure 2. Total cell protein (TCP) and enzyme protein (EP) concentration on various fermentation media during the production of L-glutaminase from *Vibrio azureus* JK-79.

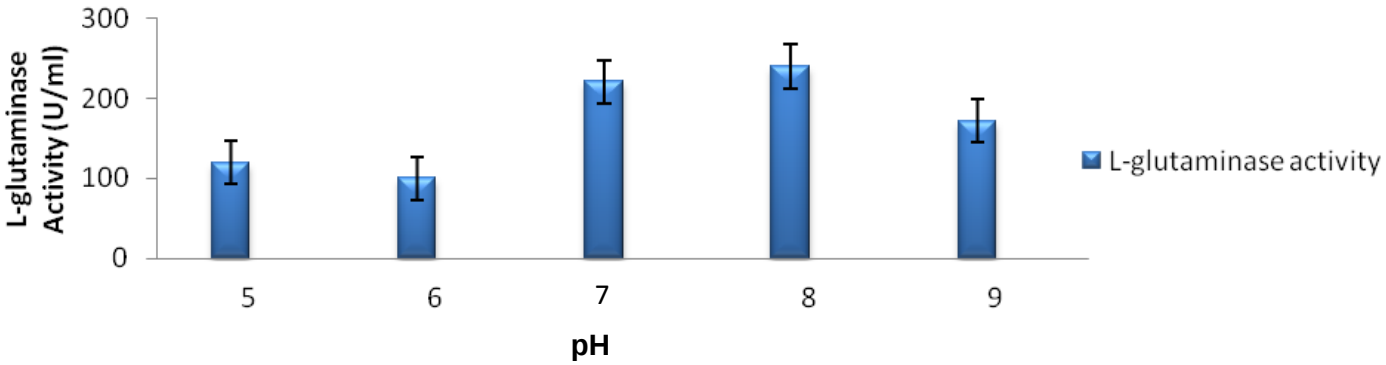

Figure 3. Effect of pH on enzyme production at 37°C and 120 rpm.

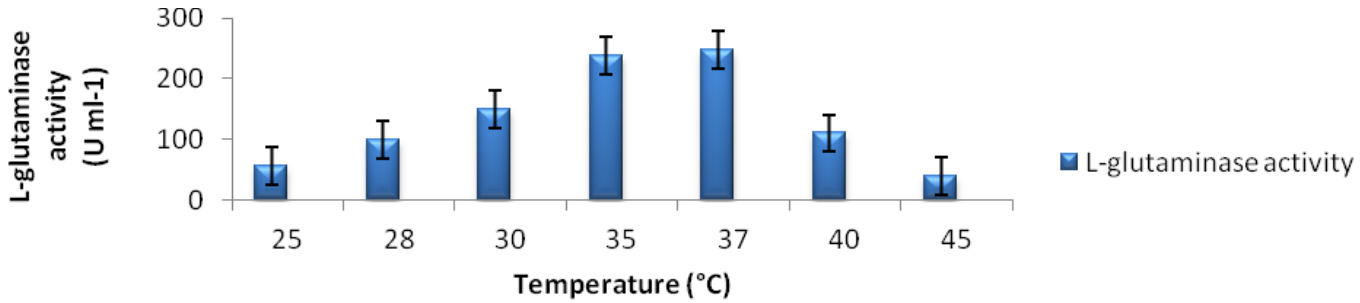

Figure 4. Effect of temperature on L-glutaminase production from *Vibrio azureus* JK-79 at pH 8 and 120 rpm.

quent metabolic product formation. In general, L-glutaminase production by most of the microbial organisms under submerged fermentation conditions is observed to be optimum in the pH range 5.0 to 9.0. The results presented in Figure 3 indicate that pH of the fermentation medium influence the enzyme production. Thus maximal enzyme production was observed at pH 8.0 (241.56 U/ml). Either increase or decrease in the pH of the medium resulted in decreased enzyme production. Results also suggest this marine bacterium is alkalophilic in nature.

Effect of temperature

Incubation temperature influenced the rate of L-glutaminase production by marine *V. azureus* JK-79. Thus the maximal enzyme production (242 U/ml) was observed at 37°C. Variation in temperature in either way resulted in decrease of L-glutaminase production (Figure 4). The loss of activity is more at higher temperature when compared to the lower temperatures. Based on the literature, the optimum temperature for L-glutaminase production is varied with micro-organism used. It was

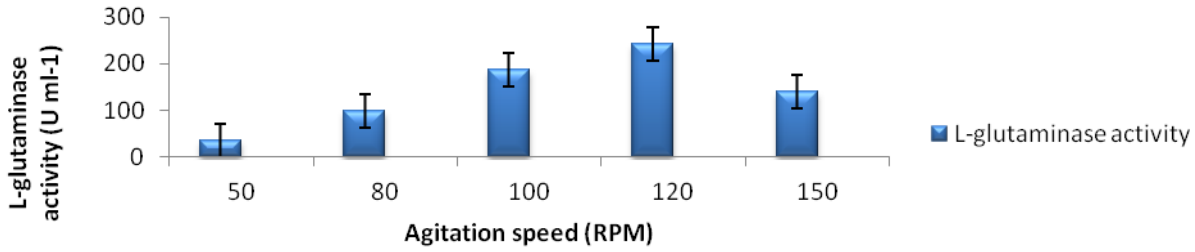

Figure 5. Effect of agitation speed on enzyme production at 37°C and pH 8.

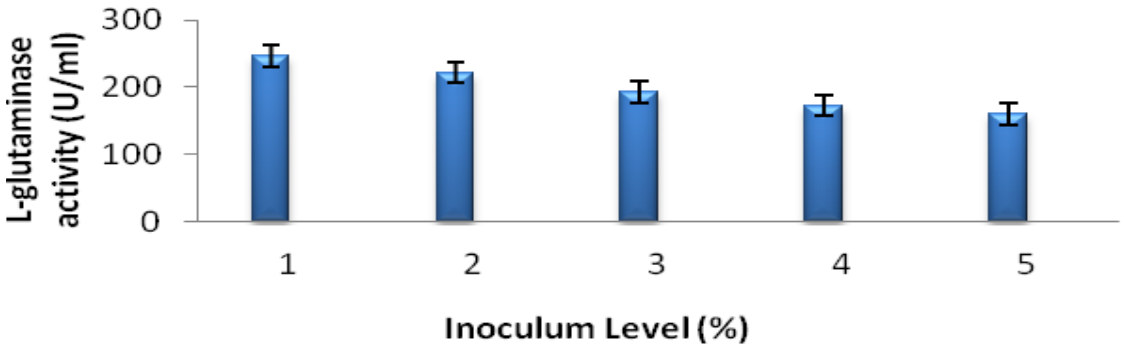

Figure 6. Effect of different inoculums levels on enzyme production at optimal conditions of pH 8, 120 rpm and 37°C.

observed that 27°C was optimum for the enzyme production by *Beauveria* sp. BTMP S10 (Keerthi et al., 1999) and *Streptomyces rimosus* (Siva Kumar et al., 2006).

Effect of agitation

The initial inoculums size controls the kinetics of growth and several metabolic functions leading to overall biomass and extracellular product formation (Subba et al., 2009). To evaluate same, experiments were planned with increasing inoculums concentration from 1 to 5%. The results (Figure 5) indicate that the kinetics of L-glutaminase production varied with variation in inoculums concentration. The maximal enzyme production (246 U/ml) was observed at 1% initial inoculums supplementation.

Effect of inoculum size

The initial inoculum size controls the kinetics of growth and several metabolic functions leading to overall biomass and extracellular product formation (Subba et al., 2009). To evaluate same, experiments were planned with increasing inoculums concentration from 1 to 5%. The results (Figure 5) indicated that the kinetics of L-glutaminase production varied with variation in inoculums concentration. The maximal enzyme production (246 U/ml) was observed at 1% initial inoculums supplementation.

Effect of incubation period

After optimization of various process parameters, a time course experiment was carried out to monitor the rate of L-glutaminase production by this novel isolate. Results presented in Figure 6 evidence that L-glutaminase production increase progressively until 24 h, when maximal enzyme production (246.86 U/ml) was recorded. The observed is in accordance with literature reports, where it is noticed that the optimum incubation period for marine *V. costicola* (Nagendra Prabhu and Chandrasekaran, 2003) were also within 24 h.

Effect of additional carbon sources

Incorporation of additional carbon sources into the enzyme production medium at 1% level, resulted in a significant increase in the enzyme production compared to the control (158 U/ml), which contained only glutamine (Figures 7 and 8). Among the various carbon sources tested, maltose supported maximal enzyme yield (301 U/ml), biomass (0.576 g/l), total cell protein (1020 µg/ml) and enzyme protein (240 µg/ml).

All the other carbon sources also showed considerable

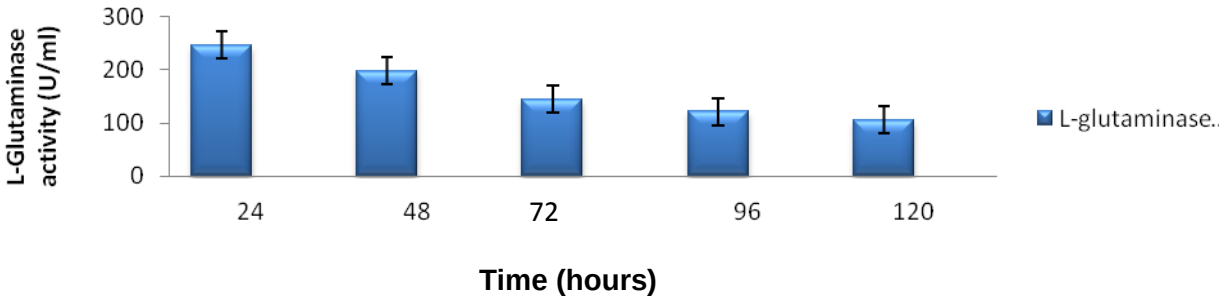

Figure 7. Effect of incubation period on enzyme production.

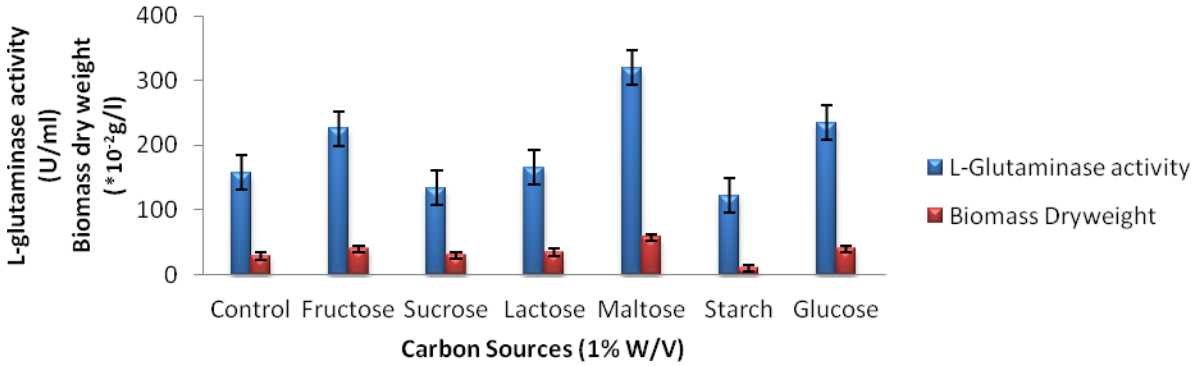

Figure 8. Effect of additional carbon sources on the enzyme production

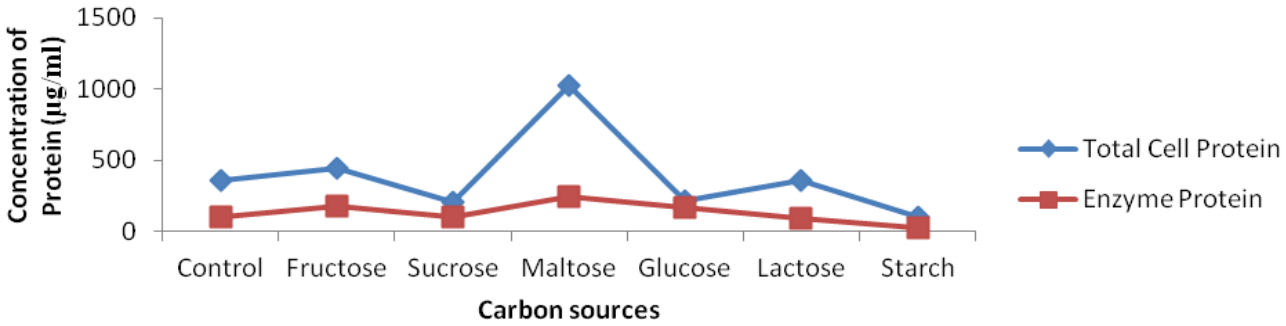

Figure 9. Concentration of TCP and EP in different carbon sources.

amount of enzyme production. Starch was the least effective as a carbon source (122 U/ml). From the result, it was observed that all the other carbon sources except starch (0.102 g/l) supported biomass production, indicating that starch could act as a growth repressor for this marine bacterial strain. The optimum concentration of maltose for maximal enzyme synthesis (321.34 U/ml) was determined to be 1.5% (Figure 9).

Effect of additional nitrogen sources

Incorporation of additional nitrogen sources, along with glutamine in the enzyme production medium influenced the rate of L-glutamine production by marine *V. azureus* JK-79. From the results presented in Figures 10 and 11, it was inferred that among the organic nitrogen sources tested, soybean meal supported maximum enzyme production (226 U/ml), total cell protein (710 µg/ml) and enzyme protein (360 µg/ml). Further studies on optimization of soybean meal indicated that 2% (w/v) was optimal for maximal enzyme production (289 U/ml) and further increase in the concentration of soybean meal resulted in decrease in enzyme production (Figure 12). From the result (Figure 13), it was observed that none of the inorganic nitrogen sources promoted the enzyme

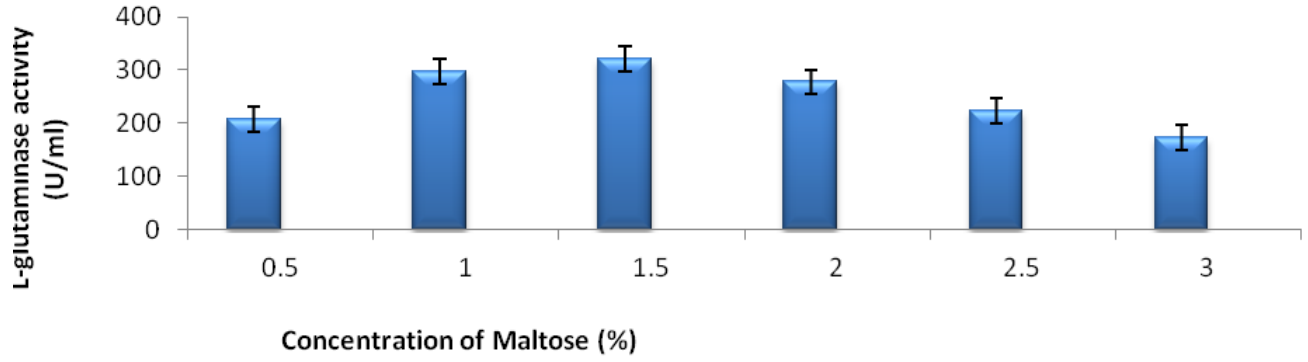

Figure 10. Concentration of maltose on enzyme production.

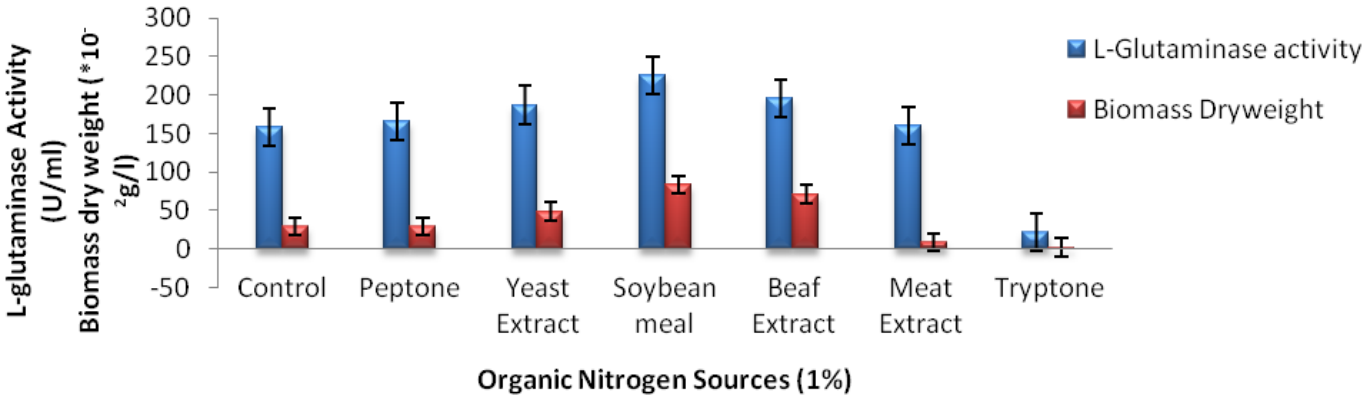

Figure 11. Effect of additional nitrogen sources on enzyme production.

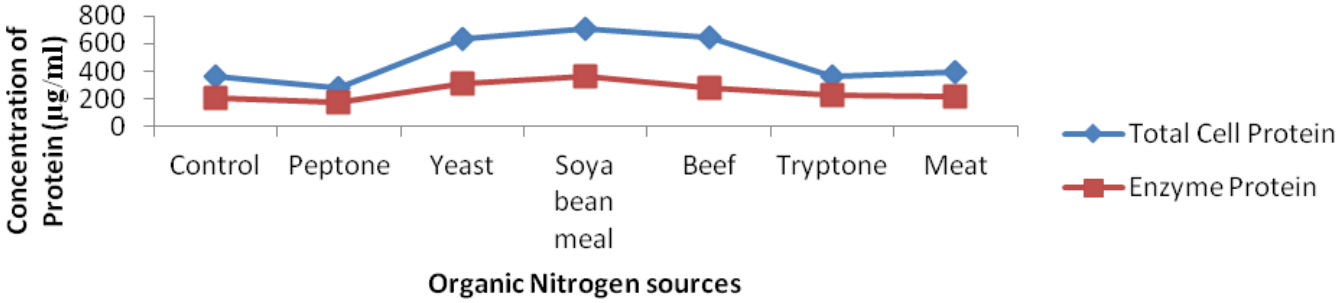

Figure 12. Concentration of TCP and EP on additional nitrogen sources.

synthesis as compared to organic nitrogen source.

Effect of amino acids

Need for amino acids as inducer compound for enhanced enzyme production was evaluated by incorporating different amino acids along with carbon source (D-glucose) in the enzyme production medium. The results presented in Figures 15 and 16 show that L-glutamine supported enhanced level of enzyme production (248 U/ml), total cell protein (195 µg/ml) and enzyme protein (164 µg/ml). None of the amino acids supported either biomass growth or the enzyme production. Since L-glutamine promoted maximal enzyme production compared to other amino acids, optimal concentration of L-glutamine was evaluated. From the results (Figure 17), it was evident that 2% concentration promoted maximal enzyme

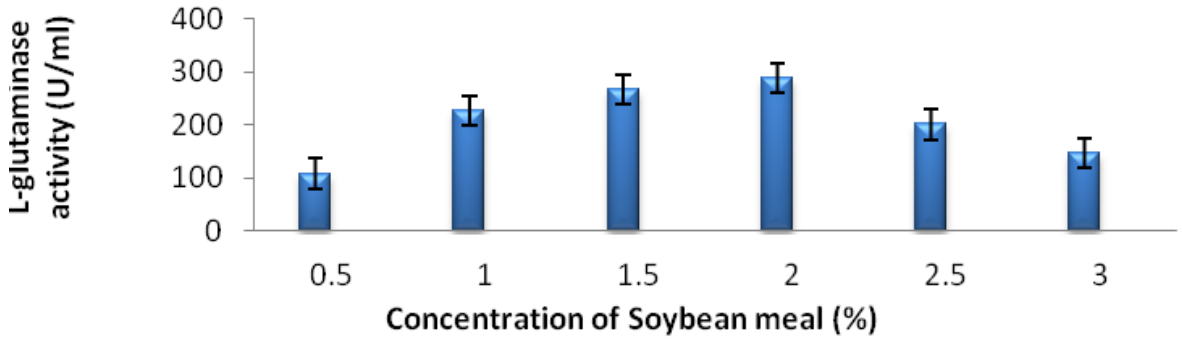

Figure 13. Effect of different concentration of soybean meal on enzyme production.

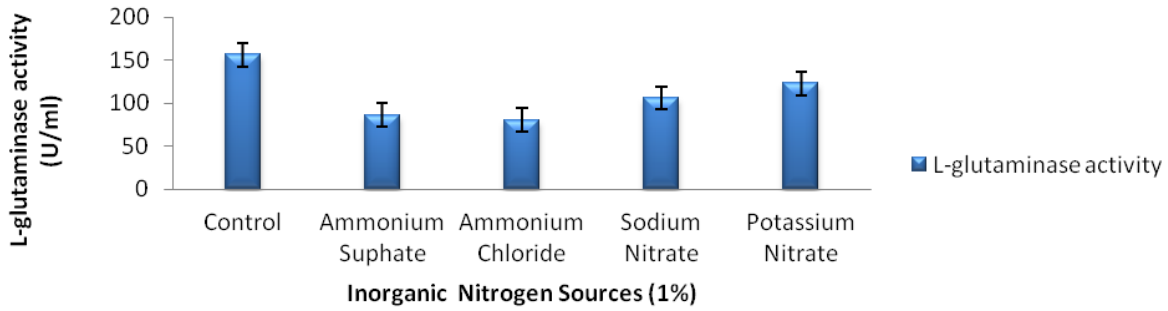

Figure 14. Effect of different inorganic nitrogen sources on enzyme production.

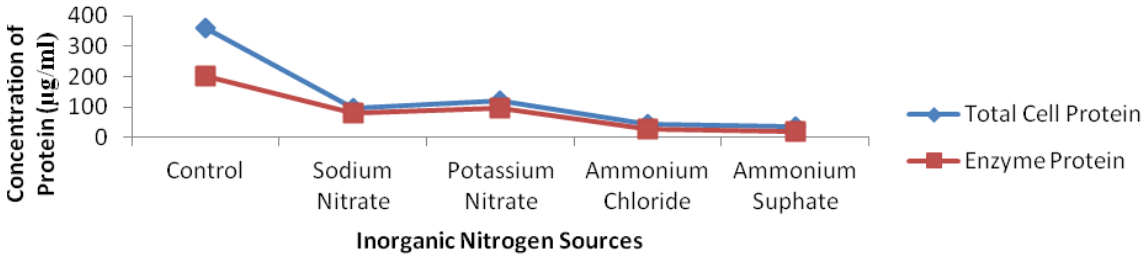

Figure 15. Concentration of TCP and EP on different inorganic nitrogen sources.

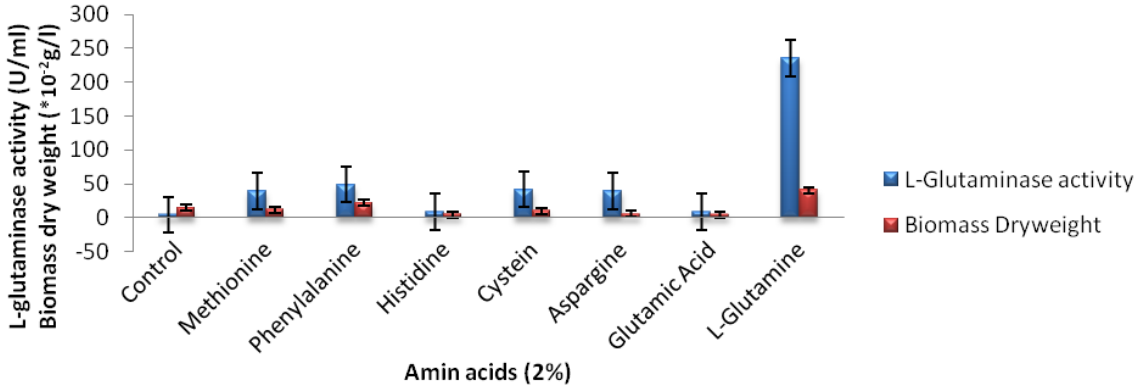

Figure 16. Effect of amino acids on enzyme production.

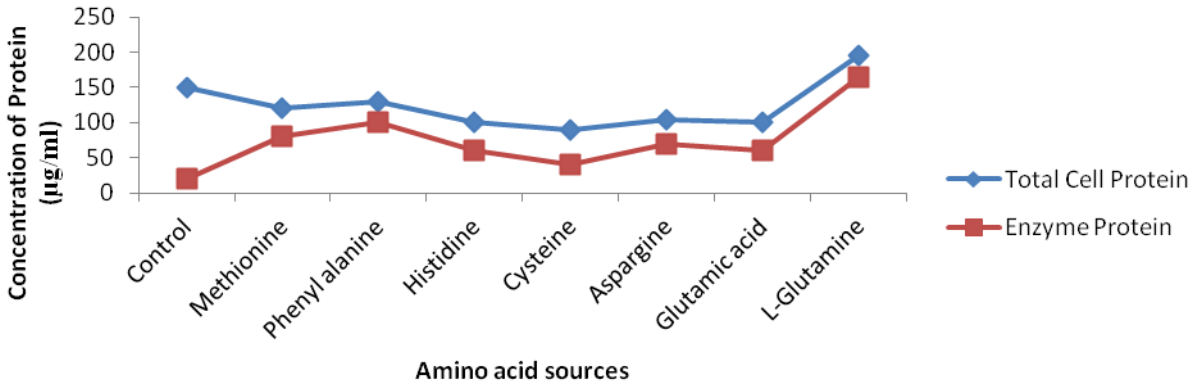

Figure 17. Concentration of TCP and EP on various amino acids.

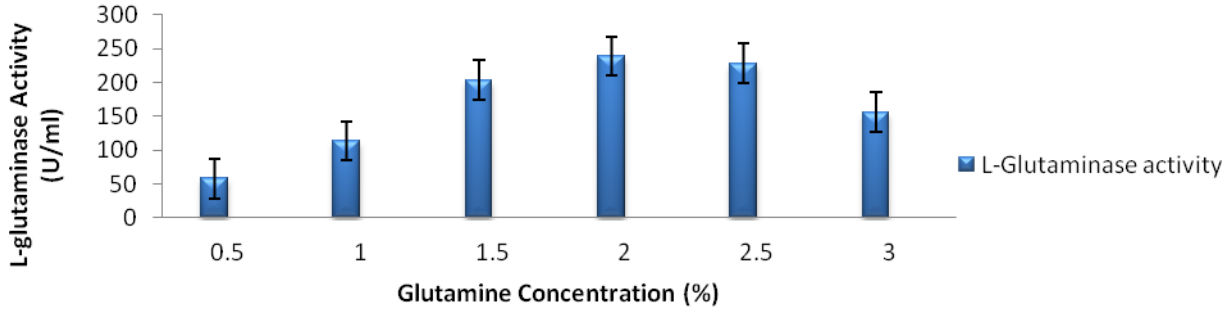

Figure 18. Effect of different glutamine concentration on enzyme production.

production (249 U/ml). Further, in general concentration upto 2% were observed to support the enhanced enzyme production.

Conclusion

The potential of isolated novel strain *V. azureus* JK-79 for L-glutaminase production was analyzed under sub-merged fermentation with different process parameters and medium constituents. Maximum production was noticed at pH 8, 37°C, 120 rpm, with 1% inoculums size, 2% glutamine concentration, 1.5% maltose, 2% soybean meal and 24 h of incubation period. Under optimal conditions, the glutaminase production improved to 321 U/ml. The results of the present study indicate this novel strain has immense potential as an industrial organism for the production of L-glutaminase as extracellular enzyme employing submerged fermentation. Further, it was observed that from the course of the present study, sea water could be used as an ideal fermentation medium for L-glutaminase production.

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Full Length Research Paper

Preliminary spectroscopic characterization of PEGylated Mucin

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The local textile industry in Itokun village is one major textile industry in Abeokuta Ogun state, known for "adire" production whose processes are not maintained at regulatory standards. This study involves isolating and identifying aerobic microorganisms in waste water effluents from this textile Industry and screening for aerobic degraders among the isolates. Fifty milliliter (50 ml) of three dye effluents; purple, brown and green were collected into sterile bottles and designated (Ef_1), (Ef_2) and (Ef_3). Tap water was used as control (Cont). Isolation, identification and screening of isolates for percentage decolorization were done using standard method. Colony forming units showed the trend Ef3 > Ef2> Ef1. Bacterial isolates included *Bacillus megaterium, Staphylococcus simulans, Micrococcus varians, Bacillus niacinni, Lysinibacillus* sp. and *Bacillus carbonificus*. *Aneurinbacillus aneurinilytus* alone was isolated from the control sample. *Bacillus megaterium* and *Lysinibacillus* sp. were the most predominant species in the effluents. *S. simulans* degradation was the most efficient and its use could be exploited.

Key words: Aerobic bacteria, textile effluent, dye, Itokun.

INTRODUCTION

Every day, industrial, commercial and domestic activities produce wastes; some of which are hazardous to the health of the general public and the ecosystem. USEPA (2005), listed seven major hazardous constituents of dye as aniline, o-anisidine, 4-chloroaniline, p-cresidine, 1,2-phenylenediamine, 1,3-phenylenediamine, and 2,4-dimethylaniline.

Dyes are synthetic aromatic compounds with various functional groups. They are used extensively in textile industries (for dyeing clothing materials), paper printing, colour photography, pharmaceutical industries, food industries, cosmetic industries, leather and other Industries (Rafi et al., 1990; Ola et al., 2010). Approximately 10,000 different dyes and pigments are used industrially, and almost one million tons of synthetic dyes are produced annually, worldwide (Olligaard et al., 1999; Adebayo et al., 2004). Coloured dye effluents are enormously toxic to the aquatic ecosystem. They change the symbiotic process by disturbing the natural balance through reduced photosynthetic activity due to the colouration of the water in streams and other water bodies. With the increased use of a wide variety of these dyes, pollution by dye wastewater is becoming increasingly startling hence, serious environmental damages are inevitabe (Cunningham and Saigo, 2001; Padmavathy et al., 2003; Asamudo et al, 2005).

Biodegradation is the breakdown of contaminating compounds (toxic) into simpler, less toxic or non toxic forms using microorganisms (Prescott et al., 2008). These microorganisms (usually fungi and bacteria) often

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use contaminants as their food source, thereby, completely eliminating toxic compounds breaking them into basic (non toxic) elements such as carbondioxide and water (mineralization). Incomplete degradation may also occur; which is the partial breakdown of the original contaminant to a less complex form. Typically, biodegradation provides an efficient and economic way to reduce environmental hazardous wastes using indigenous or introduced microorganisms that naturally degrade these contaminants. Several members of Algae, Fungi and Bacteria are known to be able to degrade various compounds and toxic wastes. These microorganisms are capable of transforming or degrading a variety of organic and inorganic contaminants such as perchlorate, radionuclides, lead, mercury, petroleum products, arsenic, etc., at levels below possible health risk standards (Wu et al., 2001; Okeke et al., 2002; Wang, 2008, 2009).

Bacillus spp*. and Paenibacillus* spp. have been isolated as degraders from dye effluent Shanooba et al. (2011). *B. brevis, B*. *formosus*, and *B. chosinensin* strain is also reported to be good degraders of Toluidine blue dye (Alhassani, 2007).

Bacillus spp. was reported by Modi et al. (2010) to be very effective in the decolourization of sulfonated dye after 72 h. *B. cereus* was reported to be potent for the decolourizing sulfonated azo dyes while *B. megaterium* had shown degradation of red 2G by 64.89% percentage (Khan, 2011). *Staphylococcus simulans* amongst other *Staphylococcus* species and microbial flora of the human body had caused degradation and decoulorisation of various dyes. *S. simulans* (NCH298) caused 100% degradation in 1.25 hrs of MR (Methyl red) and 86% deduction in 24 hours of OrII (Orange II) (Stingley et al., 2010).

The local textile industry in Itokun village is a major textile industry in Abeokuta south local government area, known for “adire” production and sales. The production processes (majorly manual) as well as effluent treatment are not maintained at regulatory standards. Their operations pose a potential risk to the community as some of the dye constituents are carcinogenic to human beings and toxic to the aquatic habitat, therefore, the degradation and/or decolourization of these azo dyes have become a necessity.

The research into bioremediation of pollutants is receiving plausible response in recent times and information on degraders among bacterial isolate have not been reported from this site, an industry without regulatory standards. The aim of this study was to isolate and identify aerobic microorganisms in waste water effluents from Itokun Textile Industry, Abeokuta, Ogun State and to screen for degraders among the isolates. The objectives of this study were to isolate and enumerate aerobic microorganisms present in dye samples as well as determine the degraders among the isolates.

MATERIALS AND METHODS

Sample collection

Fifty milliliter (50 ml) wastewater of different colours; purple (EF_1), brown (EF_2) and green (EF_3) were collected aseptically into 3 sterile sample bottles from three different points at Itokun Textile Industry Abeokuta. Collected dye effluents were placed in a brown envelope and transported to the laboratory, and tap water was used as control (Cont). Serial dilutions of the solution (10^{-1} to 10^{-9}) were done.

Bacterial cultures

One milliliter (1.0 ml) from diluents 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were aseptically inoculated unto nutrient agar (NA) plates using the spread plate method and incubated at 37°C for 24 h. Un-inoculated NA plates were also incubated to serve as the controls. This was done in duplicate and distinct colonies were enumerated using colony counter and distinct colonies were sub- cultured to obtain pure cultures. Pure cultures were further subjected to identification processes. Pure cultures were transferred into slants of Nutrient agar in Bijou bottles and preserved at 4°C in the refrigerator.

Microscopic and biochemical characterization of bacterial isolates

This included standard identification procedure like Gram-staining procedure described by Brown (2007) and biochemical tests including, oxidase test (Cheesbrough, 2006a), catalase test, (Cheesbrough, 2006b), coagulase test (Goldstein and Roberts, 1981), motility test (Perilla, 2003), Indole test, (Maria, 2010), Urease test, hydrogen sulphide production (triple sugar iron) (TSI) test, methyl red vogues proskauer test and Citrate Utilization test (Claus, 1989). Final identification of isolates was done by software (ABIS online- www.abis.online.com).

Screening for aerobic dye degrader

0.015 g of each dye RB 13(Red), RY(Yellow) and RR (Red) was put in a 500 ml conical flask, 500 ml of distilled water was added and the dye mixture was designated (RYR). The dyes were allowed to dissolve properly, shaken and autoclaved at a temperature of 121°C for 15 min. A sterile pipette was used to dispense 5 ml of the dye into sterile test tubes, including the control. The initial absorbence (A_0) was determined using the UV visible spectrophotometer machine. Afterwards, 5 ml of each pure bacteria grown overnight in nutrient broth was aseptically transferred into the tubes of dye mixture (RYR); each tube was sealed with sterile cotton wool and labeled accordingly. The test tubes were incubated for 24 h after which they were centrifuged and the absorbance of the supernatant was measured (At) using UV visible Spectrophotometer machine (Olukanni et al., 2010), all set-up was done in triplicate.

Data analysis

Data collected are presented with descriptive statistics.

RESULTS AND DISCUSSION

Bacterial count

The colony forming units were counted and calculated for

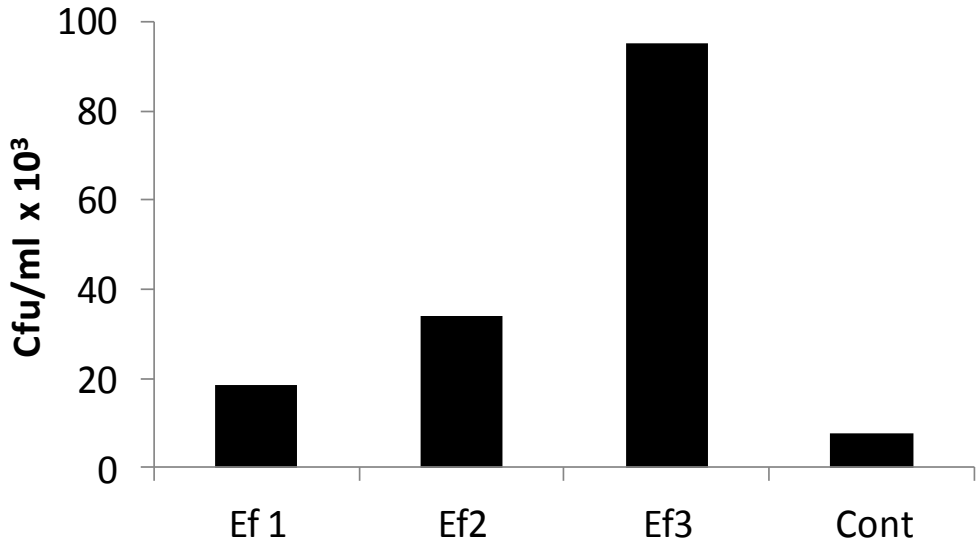

Figure 1. A bar chart representing the number of colony forming unit in effluents samples and control. Ef1 = Purple; Ef2 = Brown; Ef3 = Green; Cont = Tap water control; Cfu/ml = Colony forming units/milliliters.

the various dye samples EF_1, Ef_2 and Ef_3 (Figure 1). Ef_3 showed the highest Cfu /ml, followed by Ef_2 and then Ef_1 was the least. All effluent samples had higher colony forming unit than the control.

Identification of bacterial isolates

Pure isolates obtained from effluent samples collected from Itokun textile industry Abeokuta were identified. Each isolate was given a sample number. Gram stain and biochemical results for all samples are presented in Table 1. Table 2 shows each bacterial isolated in the different effluent samples and the control sample.

The organisms isolated from the samples were *B. megaterium, S. simulans, M. varians, B. niacinni, Lysinibacillus* sp., *B. carbonificus. A. aneurinilytus* alone was isolated from the control sample. *B. megaterium* and *Lysinibacillus* sp. were the most predominant species in the effluents. Most bacteria isolated from effluent have been reported in literatures as degraders of dye except *A. aneurinilytus.*

Three species of *Bacillus*; *B. megaterium, B. carbonificus* and *B. niacinni* wer reported in this study. Findings from Khan (2011), indicated that *B. megaterium* isolated from marina beach degrade red 2G by 64.89%. Moreover, *B. megaterium* NCIM 2087 and *P. desmolyticum* NCIM 2112 showed 70% decoulorisation of textile dye Vat Blue 66. *Bacillus* spp. had earlier been isolated from dye effluent (Shanooba et al., 2011) and three strains of *B. brevis, B*. *formosus*, and *B. chosinensin* strain had earlier been reported to be good degraders of Toluidine blue dye (Alhassani, 2007). *Bacillus* spp. was reported to be more effective for decolourization of sulfonated dyes; 95% of reactive red 195 dye (Modi et al., 2010). However, *B. megaterium* identified in this study showed only 11.3% decolorization in the dye mixture (RYR) in 24 h, and this could be due to the mixture of dye used. Lysinibacillus spp. showed the lowest degradation (0.27%) from this study, although Dawker (2007) had reported that *Bacillus* spp., and other Lysinibacillus *spp* were used for the degradation of dye*. A. aneurinilyticus,* among other bacteria were isolated from hot spring waters of kharaghan (in Gazvin province) and Mahallat (in Arak). *A. aneurinilyticus* was also isolated in this study from the control water sample.

S. simulans was isolated in this study and it could be as a result of the manual method of textile processing employed in this industry which involves the use of hands; this bacterium amongst other *Staphylococcus* species are microbial flora of the human body had been demonstrated to cause degradation and decoulorisation of various dyes.

Percentage decolorization of dye mixture

The percentage decolorization of dye mixture (RYR) is presented in Table 3. The result indicates that *Lysinibacillus* sp. showed the lowest percentage decolorization (0.27%) followed by *M. varians* (8.5%) while the highest decolorization was recorded by *S. simulans* (88.9%) followed by *B. niacinni* (23.3%).

S. simulans which showed 88.98% decolorization of dye mixture (RYR) in 24 h from this study caused 100% degradation in 1.25 h of MR (Methyl red) and 86% deduction in 24 h of OrII (Orange II) (Stingley et al., 2010). Variation in the percentage degradation of dye

Table 1. Identification of Isolated bacterial organism.

Isolate number	Catalase	Oxidase	Methyl red	Motility	Voges -Proskauer	Citrate	Urease	Indole	Gram reaction	Shape	Coagulase	Suspected organism
1	+	-	-	+	-	+	+	-	+	Rods	-	*Bacillus megaterium*
2	+	-	-	+	-	-	+	-	-	Cocci	-	*Staphylococcus simulans*
3	+	+	+	-	-	+	+	+	-	Rods	-	*Aneurinibacillus aneurinilytus*
4	+	-	-	+	-	+	-	-	+	Cocci	+	*Micrococcus varians*
5	+	-	-	-	-	-	-	+	+	Rods	-	*Bacillus carboniphilus*
6	+	-	-	-	-	-	-	+	-	Rods	-	*Bacillus niacinni*
7	+	-	+	+	-	-	+	+	+	Rods	-	*Lysinibacillus* sp.

Table 2. Bacterial isolates from the effluent samples and control.

Sample	Species found (micro organism)
Ef_1	*Micrococcus varians*, *Bacillus megaterium*
Ef_2	*Lysinibacillus, Baccillus megaterium, Bacillus carboniphilus*
Ef_3	*Staphylococcus simulans, Bacillus niacinni, Lysinibacillus*
Control	*Aneurinbacillus aneurinilytus*

Ef_1 = Purple; Ef_2 = Brown**;** Ef_3 = Green.

Table 3. Percentage decolorization of dye (RYR) using isolates.

Isolate	Average absorbance	% Absorbance
Micrococcus varians	0.68±0.01	8.514
Bacillus carbonificus	0.58±0.01	21.081
Bacillus niacinni	0.57±0.03	23.378
Lysinibacillus sp	0.74±0.02	0.270
Bacillus megaterium	0.66±0.03	11.351
Staphylococcus simulans	0.08±0.02	88.986

$$\% \text{ Decolourization} = \frac{A_0 - A_t}{A_0} \times 100$$

Where, A_0 is the 0.740 nm; A_0 is the Initial absorbance; At is the final absorbance; Rep is the representative.

with *S. simulans* could be due to the mixture of dyes used. This study therefore supports that *S. simulans* is a good degrader of dye.

Conclusion and recommendation

Seven aerobic bacteria were isolated from the Itokun textile effluent an indication of the hostile condition for the normal flora of water. *S. simulans* proved to be the most efficient degrader of the dye mixture (RYR), its mechanism and best condition of degradation could be researched into and its use as a degrader could be exploited. The textile industry in Itokun though informal has great potential to be exploited by government and should be institutionalized. With government intervention, monitoring the industry's activities and processes could be embarked on and policy which ensures proper treatment of their effluents before discarding into neighbouring environments could then be enacted and executed.

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Optimization of culture variables for the production of L- asparaginase from *Pectobacterium carotovorum*

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L-Asparaginase is an anti-neoplastic agent used in the chemotherapy of acute lymphoblastic leukemia. The present work deals with production of intra-cellular L-asparaginase from *Pectobacterium carotovorum* using submerged fermentation. The statistical approaches of Plackett-Burman and response surface methodology were applied to optimize the culture parameters for the production of L-asparaginase through a bacterium *P. carotovorum* MTCC 1428. Optimal conditions for maximal productivity were determined based on six parameters at two different levels. Initially, a screening design methodology was used to evaluate the process variables, and the response surfaces applied to find optimal concentration for production. The analysis revealed that the optimum levels of L-asparagine, lactose and inoculum percentage were found to be 4.00, 3.5 g L^{-1}, and 2.75%, respectively. The maximum enzyme activity in the optimized medium was 19.33 U ml^{-1}.

Key words: Design of experiments (DoE), L-asparaginase, response surface methodology, Plackett-Burman design and acute lymphoblastic leukemi.

INTRODUCTION

L-Asparaginase (L-asparagine amido hydrolase E.C. 3.5.1.1) is an enzyme of high therapeutic value due to its use in certain kinds of cancer therapies, mainly in acute lymphoblastic leukemia (ALL) (Athale, 2003; Narta et al., 2007 and Mishra, 2007). It is also used in food industry for the production of acrylamide free food (Pedreschi et al., 2008), model enzyme for the development of new drug delivery system (Teodor et al., 2009) and L-asparagine biosensor for leukemia (Verma et al., 2007). Studies on the molecular structure (Aung et al., 2007), catalysis (Kleo et al., 2002), clinical aspects (Narta et al., 2007), crystal structure (Yun et al., 2007), genetic determinants involved in regulation (Huser et al., 2002) and stabilization to enhance biological half-life (Ofagain, 2003) of L-asparaginase have been reported.

The anti-leukemic effect of L-asparaginase is postulated to result from the rapid and complete depletion of the circulating pool of L-asparagine, as most of the cancer cells are dependent on an exogenous source of this amino acid for survival. However, normal cells are able to synthesize L-asparagine and thus are less affected by its rapid depletion due to treatment with this enzyme (Fisher and Wray, 2002). The L-asparagine deficiency rapidly impairs the protein synthesis and leads to a delayed inhibition in DNA and RNA synthesis and hence an impairment of cellular functions, resulting in cell death (Narta et al., 2007; Muller, 1998).

The production of L-asparaginase has been studied in *Serratia marcescens* (Heinemann and Howard, 1969), *Erwinia carotovora* (Howard and Carpenter, 1999), *Escherichia coli* (Neu and Heppel, 1965; Kenari et al., 2011), *Enterobacter aerogenes* (Geckil and Gencer; 1982, Baskar, 2011), *Pseudomonas aeruginosa* (Abdel-Fattah and Olama, 2002), *Bacillus subtilis* (Fisher and Wray,2002)*Bacilluscereus*(Sunithaetal.,2010),*Aspergillus tamari*, *Aspergillus niger*, *Aspergillus terreus* (Kelo et al.,

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2002, 2004; Theantana et al., 2007; Siddalingeshwara et al., 2011), and *Streptomyces noursei* MTCC 10469 (Dharmaraj, 2011), and *Yersinia pseudotuberculosis* (Abakumova et al., 2009), with various carbon and nitrogen sources. The synthesis of L-asparaginase is regulated by environmental and nutria-tional factors. In most of the L-asparaginase fermentation processes, the presence of partial glutaminase activity up to 9% of L-asparaginase activity was reported (Muller and Boss, 1998). The various side effects of this drug are mainly due to the presence of partial glutaminase activity (Distasio et al., 1982). Therefore, glutaminase-free L-asparaginase is essentially required for successful clinical studies (Devi et al., 2012; Roberts et al., 1972; Ramya et al., 2012).

In developing a biotechnology-based industrial process, designing the fermentation media is of critical importance. The fermentation medium affects the product yield and volumetric productivity. It is also important to reduce the cost of the medium as much as possible, as this may affect the overall process economics. Medium screening studies are very time consuming and expensive. For economy of effort and scale, different approaches have been used to rapidly identify the variables, which need to be controlled for optimizing production of useful metabolites. However, the conventional methods of optimizing medium composition via sequential manipulation of single parameter often fail to identify the optimal conditions for the bioprocess because interactions between different factors are neglected.

The Plackett-Burman factorial designs allow for the screening of main factors from a large number of process variables, and these designs are thus quite useful in preliminary studies in which the principal objective is to select variables that can be fixed or eliminated for further optimization processes (Araujo and Brereton, 1996). Response surface methodology is a well-known method applied in the optimization of medium constituents and other critical variables responsible for the production of biomolecules (Khuri and Cornell, 1987). Thus, optimum performance has been determined using mathematical tools such as multiple regression of a partial or full factorial to obtain a model of the production system, usually involving fitting of data to a polynomial equation, using stepwise multiple regression. However, several interacttions of the experimental design and optimization of models are required for effective application to product formation in fermentation systems.

In the present investigation, the production of extracellular asparaginase from *Pectobacterium carotovorum* MTCC 1428 was enhanced by optimization of culture parameters. The optimization of the bioprocess was carried out through a stepwise experimental strategy including: (1) screening the most significant factors affecting enzyme production using a two-level multi-factorial Plackett-Burman design, (2) optimization of the most significant components and generating a mathematical model expressing the relationship between optimized factors and asparginase production by application of central composite design and (3) verification of the model at both shake flask and fermenter scale.

MATERIALS AND METHODS

Microorganism

The bacterium used throughout the study, *P. carotovorum* MTCC 1428, was procured from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India.

Activation and culture conditions

This organism was grown on the medium containing (g/L): beef extract, 1.0; yeast extract, 2.0; NaCl, 5.0; peptone, 5.0; and agar, 15.0 (pH 7.0) at 30°C. The organism was sub-cultured every month and maintained at 4±1°C. The production of L-asparaginase was studied in the modified basal semi-synthetic medium containing (g/L): glucose, 3; $Na_2HPO_4 \cdot 2H_2O$, 6.0; KH_2PO_4, 3; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.015; yeast extract, 1.0; and peptone, 1.0 with an initial pH of 6.5 (Gulati et al., 1997). The inoculum was prepared by adding a loop full of freshly prepared pure culture of slant into 50 ml of previous-said autoclaved medium containing glucose as carbon source in a 250 ml shake flask and incubated at 30°C and 180 rpm in a shaking incubator for 10 to 12 h (to reach culture OD at 600 nm=0.6 to 0.8). 2% of the inoculum from the above-said culture was added to 50 mL of the medium in 250 mL shake flasks. The flasks were incubated in a shaking incubator at 30°C and 180 rpm. Samples were withdrawn at regular interval of time and measured for L-asparaginase production. Experiments were conducted in duplicates and enzymatic assay was performed in duplicates for each sample.

Assay of L-asparaginase and L-glutaminase

Samples were centrifuged at 7,000 × *g* for 10 min at 4±1°C and washed twice with 0.05 M Tris–HCl buffer (pH 8.6) and ultrasoniccated (SONICS Vibra cell, USA) at 20 kHz, 35% amplitude, four cycles (2 min per cycles with 2 s on and 1 s off). The contents were centrifuged at 10,000 ×*g* for 10 min (4±1°C) and the supernatant was analyzed for intracellular L-asparaginase activity by the modified method of Wriston (1985).

L-Asparaginase catalyzes the L-asparagine to L-aspartic acid and ammonia and the latter can react with the Nessler's reagent to produce an orange product. The enzyme assay mixture consisted of 900 µl of L-asparagine (100 m M) in Tris-HCl buffer (pH 8.6) and 100 µl of crude extract of enzyme. The reaction mixture was incubated at 37°C for 30 min and 100 µl of 15% trichloroacetic acid (TCA) was added to stop the reaction.

The reaction mixture was centrifuged at 10,000 rpm for 5 min to remove the precipitates and the ammonia released in the supernatant was determined colorimetrically by adding 100 µl Nessler's reagent into sample containing 500 µl supernatant. The contents in the sample were vortexed and incubated at room temperature for 10 min and O.D. at 425 nm was measured against the blanks that received TCA before the addition of cell suspension.

The ammonia produced in the reaction was determined based on a standard curve obtained with ammonium sulfate as standard. L-Glutaminase activity was determined as described previously for L-asparaginase activity using the modified method of Wriston and Yellin (1973). One unit of enzyme activity was defined as the

Table 1. Experimental variable at different levels used for the production of L-asparaginase by *Pectobacterium carotovorum* 1428 using Plackett-Burman design.

Variable	Symbols code	Experimental value	
		Lower	Higher
Lactose (g L^{-1})	X_1	1.5	5.0
Maltose (g L^{-1})	X_2	1.5	5.0
L-asparagine (g L^{-1})	X_3	3.0	5.0
Tryptone (g L^{1})	X_4	0.5	2.5
Inoculum size (%)	X_5	1.5	5.0
Agitation speed (Rpm)	X_6	100	250

Table 2. Plackett- Burman design matrix in coded units and real values (in parenthesis) along with the observed and predicted L-asparaginase production.

Run order	Experimental value						Enzyme activity (Uml^{-1})	
	X_1	X_2	X_3	X_4	X_5	X_6	Observed	Predicted
1	1(5.0)	-1(1.5)	1(5.0)	-1(0.5)	-1(1.5)	100	15.36	15.7717
2	1(5.0)	1(5.0)	-1(3.0)	1(2.5)	-1(1.5)	100	12.84	12.5517
3	-1(1.5)	1(5.0)	1(5.0)	-1(0.5)	1(5.0)	100	11.03	10.6050
4	1(5.0)	-1(1.5)	1(5.0)	1(2.5)	-1(1.5)	250	14.85	14.5150
5	1(5.0)	1(5.0)	-1(3.0)	1(2.5)	1(5.0)	100	11.53	11.8183
6	1(5.0)	1(5.0)	1(5.0)	-1(0.5)	1(5.0)	250	13.76	14.0983
7	-1(1.5)	1(5.0)	1(5.0)	1(2.5)	-1(1.5)	250	09.96	10.0817
8	-1(1.5)	-1(1.5)	1(5.0)	1(2.5)	1(5.0)	100	10.40	10.2883
9	-1(1.5)	-1(1.5)	-1(3.0)	1(2.5)	1(5.0)	250	08.80	09.1250
10	1(5.0)	-1(1.5)	-1(3.0)	-1(0.5)	1(5.0)	250	14.29	13.8750
11	-1(1.5)	1(5.0)	-1(3.0)	-1(0.5)	-1(1.5)	250	10.21	10.1750
12	-1(1.5)	-1(1.5)	-1(3.0)	-1(0.5)	-1(1.5)	100	10.99	11.1150

amount of enzyme that liberates 1 µmol of ammonia per minute at 37°C.

Experimental design and data treatment

The optimization of medium constituents for maximization of L-asparaginase production by *P. carotovorum* MTCC 1428 was carried out in two stages.

Screening of significant medium components by the Plackett-Burman design

The Plackett-Burman experimental design was applied to screen the significant culture variables to maximize the production of L-asparaginase (Plackett and Burman, 1946). A total of six parameters, *viz*., lactose, maltose, L-asparagine, tryptone, agitation speed, and inoculum percentage have been considered for the screening experiment. Each independent variable was represented at two levels, high and low, which are denoted by (+1) and (-1), respectively. The experimental design with the name, symbol code and actual level of the variables is shown in Table 1, whereas Table 2 shows the detail of the design. Asparaginase production was carried out and enzymatic assay was taken in duplicates and the average value was taken as the response. According to the Plackett-Burman experimental design, a total of 12 experiments were performed. The significance of each variable was determined using Student's t test and the results are shown in Table 3.

Optimization of screened components by response surface methodology and statistical analysis

A 2^3 full-factorial central composite design (CCD) with three screened medium constituents that is lactose, L-asparagine and inoculum percentage was generated by Minitab statistical software Release 15. Each variable (medium component) was assessed at five coded levels (− 2, − 1, 0, +1, and +2) with 20 (= 2^k +2k + 6) treatment combinations where k is the number of independent variables. The relationships and interrelationships of the variables were determined by fitting the second-order polynomial equation to data obtained from 20 experiments. The minimum and maximum ranges of the variables were used and the full experimental plan with regard to their values in actual and coded form is provided in Table 4. The fitted equation was then expressed in the form of two-dimensional contour plots and three-dimensional response surface plots to illustrate the main and interactive effects of the independent variables on the dependent ones.

Validation of the experimental model

The experimental model was verified by performing the experiments at optimal levels of most significant variables and at the middle level of other medium components both in shake flask and in a 7.5 L bioreactor (NEW BRUNSWICK Cell Biogen Model 310). The medium was inoculated with 2.7% inoculum and the bioreactor was operated at 25°C, 100 rpm and 1.2 vvm with uncontrolled pH. The

Table 3. Statistical analysis of the Plackett – Burman design showing coefficient, *t*, and *p* values for each variable.

Variable	Symbol code	Effect	Coefficient	*t*-value	*p*-value
Intercept			12.0017	90.08	0.000
Lactose	X_1	3.5400	1.7700	13.29	0.000[b]
Maltose	X_2	-0.8933	-0.4467	-3.35	0.020[a]
L-asparagine	X_3	1.1167	0.5583	4.19	0.005[b]
Tryptone	X_4	-1.2100	-0.6050	-4.54	0.016[a]
Inoculum size	X_5	-0.7333	-0.3667	-2.75	0.004[b]
Agitation speed	X_6	-0.0467	-0.0233	-0.18	0.868[a]

s = 0.461526; r-sq = 97.90%; r-sq(adj) = 95.39%. (a) Non-significant at p>0.005; (b) significant.

Table 4. 2^3 full-factorial central composite circumscribed design matrix in coded units and real values (in parenthesis) with experimental and predicted values of L -asparaginase production.

Run order	Lactose, X_1 (gL^{-1})	L-asparagine, X_3 (gL^{-1})	Inoculum Size, X_5 (%)	Enzyme activity (Uml^{-1})		Std. error
				Observed	predicted	
1	(-1) 1	(-1) 2	(-1) 0.5	11.850	11.716	1.20
2	(+1)6	(-1) 2	(-1) 0.5	10.780	10.932	-1.37
3	(-1) 1	(+1) 6	(-1) 0.5	11.890	12.056	-1.49
4	(+1)6	(+1) 6	(-1) 0.5	10.590	10.412	1.60
5	(-1) 1	(-1) 2	(+1) 5.0	11.500	11.693	-1.74
6	(+1)6	(-1) 2	(+1) 5.0	11.480	11.329	1.35
7	(-1) 1	(+1) 6	(+1) 5.0	13.290	13.153	1.23
8	(+1)6	(+1) 6	(+1) 5.0	11.780	11.929	-1.34
9	(0) 3.5	(0) 4	(0) 2.75	18.800	19.047	-1.33
10	(0) 3.5	(0) 4	(0) 2.75	19.160	19.047	0.16
11	(0) 3.5	(0) 4	(0) 2.75	18.960	19.047	-0.47
12	(0) 3.5	(0) 4	(0) 2.75	19.330	19.047	1.52
13	(-2) 0.58	(0) 4	(0) 2.75	13.090	13.043	0.39
14	(+2)7.58	(0) 4	(0) 2.75	11.380	11.404	-0.20
15	(0) 3.5	(-2) 0.73	(0) 2.75	12.990	12.960	0.25
16	(0) 3.5	(+2) 7.26	(0) 2.75	13.720	13.727	-0.06
17	(0) 3.5	(0) 4	(-2) 0.93	11.550	11.553	-0.03
18	(0) 3.5	(0) 4	(+2) 6.42	12.774	12.774	0.22
19	(0) 3.5	(0) 4	(0) 2.75	19.150	19.150	0.33
20	(0) 3.5	(0) 4	(0) 2.75	19.150	19.150	-0.72

samples were drawn at regular intervals of time, and enzyme activity was measured in duplicates. All experiments in shake flask were conducted in duplicates and averages of the results were taken as response.

RESULTS

Production of L-asparaginase

Under unoptimized levels of medium components, the production of intracellular L-asparaginase was found to be 1.20 U ml^{-1}. There was no trace of extracellular L-asparaginase observed in the culture. This assessment of the production was confirmed through liquid medium containing phenol red.

Screening of the most significant medium components by Plackett-Burman design

The intracellular asparaginase activity of *P. carotovorum* culture at 12 h was found to be maximum. The data in Table 2 indicate that there was a wide variation of asparaginase activity from 10.21 to 15.36 Uml^{-1} in 12 trials. This variation reflected the significance of factors on the enzyme activity. The analysis of regression coefficients and *t*-value of six variables are shown in Table 3. Generally, a large *t*-value associated with a low *P*-value of a variable indicates a high significance of the corresponding model term.

The main effect of each variable upon L-asparaginase production was estimated as the difference between both

Table 5. Analysis of variance for response.

Source	DF	Seq. SS	Adj. Mean square	F value	*p* value
Regression(model)	9	219.418	24.3797	566.01	0.000
Interaction	3	1.085	0.3617	8.40	0.006
Residual error	9	0.388	0.0431		
Lack of fit	5	0.209	0.0418	0.94	0.540
Pure error	4	0.179	0.0446		
Total	19	219.856			

averages of measurements made at the high level (+1) and at the low level (−1) of that variable. On the analysis of the regression coefficients of the medium components, lactose and L-asparagine showed positive effect values, whereas maltose, tryptone, agitation speed, inoculum percentage showed negative effect values. The components were screened based on their absolute value of effects (either positive or negative) and P value below at 0.005. The absolute values of effect in Table 3 were used to indicate the relative contribution of the variable on the response. Neglecting the variables which were insignificant, the model equation for asparaginase activity can be written as:

$$Y_{\text{enzyme activity}} = 12.001 + 1.77\ 0X_1 + 0.5583\ X_3 - 0.33667\ X_5 \quad (3)$$

Where, X_1 is lactose, X_3 is asparagines and X_5 is inoculum percentage.

The P values of the components maltose, tryptone, agitation speed were above 0.005 for the production of L-asparaginase and hence considered as insignificant ones. The remaining components, L-asparagine, lactose and inoculum percentage had a P value below 0.005 and were considered to be significant ones. The levels of these screened components were optimized using the central composite experimental design for enhanced production of L-asparaginase.

Optimization of medium components by central composite design

At the end of the screening experiments by Plackett-Burman design, three factors were found to play a significant role in asparaginase production. The respective low and high levels of each variable with the coded levels in parenthesis along with the CCD design with response (Uml^{-1}) are given in Table 4. The results of the second-order response surface model fitting in the form of ANOVA are given in Table 5. To test the fit of the model equation, the regression-based determination coefficient R^2 was evaluated. The nearer the values of R^2 to 1, the model would explain better for variability of experimental values to the predicted values. The model presented a high determination coefficient (R^2 = 0.9981) explaining 99% of the variability in the response (Table 6). The coefficients of regression were calculated and the following regression equation was obtained:

$$Y_{\text{enzyme activity}} = 19.098 - 0.5019\ X_1 + 0.2349\ X_3 + 0.3736\ X_5 - 2.5973\ X_1^2 - 2.1773\ X_3^2 - 2.6198X_5^2 - 2.150\ X_1\ X_3 + 0.1050\ X_1\ X_5 + 0.2800\ X_3\ X_5 \quad (4)$$

Where, X_1 is lactose, X_3 is asparagines and X_5 is inoculum percentage.

The statistical significance of previous equation was checked by *F* test and the results of ANOVA are shown in Table 5. The results demonstrate that the model is highly significant and is evident from Fischer's *F*-test with a very low probability value (*P* model > *F* = 0.0000) (Table 5). Model coefficients estimated by regression analysis for each variable is shown in Table 6. The significance of each coefficient was determined by *t*-values and *P*-values. The results reveal that all three components have a significant effect (*P* < 0.0000) on asparaginase production. The *P*-values (< 0.0001) and lack of fit (0.006) for the model suggested that the obtained experimental data were in good fit. Also, the effect of interactions of lactose with L-asparagine and L-asparagine with inoculum percentage is significant on the production while there is less interactive effect of lactose with inoculum percentage.

Two dimensional contour plots and three-dimensional response surface plots were constructed by plotting the response (enzyme activity) on the *Z*-axis against any two independent variables, while maintaining other variables at their median levels shown in Figures 1a, c and 2a, c, respectively. The response surfaces having circular contour plot indicate less interaction, whereas, an elliptical or saddle nature of the contour plot indicates significant interaction between the corresponding variables. With increase in concentration of lactose beyond certain limit, surface plot sharply descended indicating a less production of enzyme activity. Similar descending nature of surface plot was observed with asparagine as beyond 4 g/L of yeast extract; the surface tends to decline indicating a decrease in enzyme activity. This proves interaction between lactose and asparagine. Figure 1b shows that, with the increase in aspargine concentration to mid range, the surface ascended indicating enhancement of enzyme activity and beyond the mid range, the surface curvature declined but no significant change in surface

Table 6. Model coefficient estimated by multiple linear regressions.

Model term	Coefficient	Std. error (coefficient)	*t* -value	*p* value
Constant	19.0984	0.08493	224.867	0.000
X_1	-0.5019	0.05684	-8.831	0.000
X_2	0.2349	0.05684	4.133	0.003
X_5	0.3736	0.05684	6.573	0.000
X_1^2	-2.5973	0.05711	-45.479	0.000
X_2^2	-2.1773	0.05711	-38.125	0.000
X_5^2	-2.6198	0.05711	-45.873	0.000
X_1*X_2	-0.2150	0.07338	-2.930	0.017
X_1*X_5	0.1050	0.07338	1.431	0.186
X_2*X_5	0.2800	0.07338	3.816	0.004

S = 0.2075; r-sq = 99.8%; r-sq (adj.) = 99.6%.

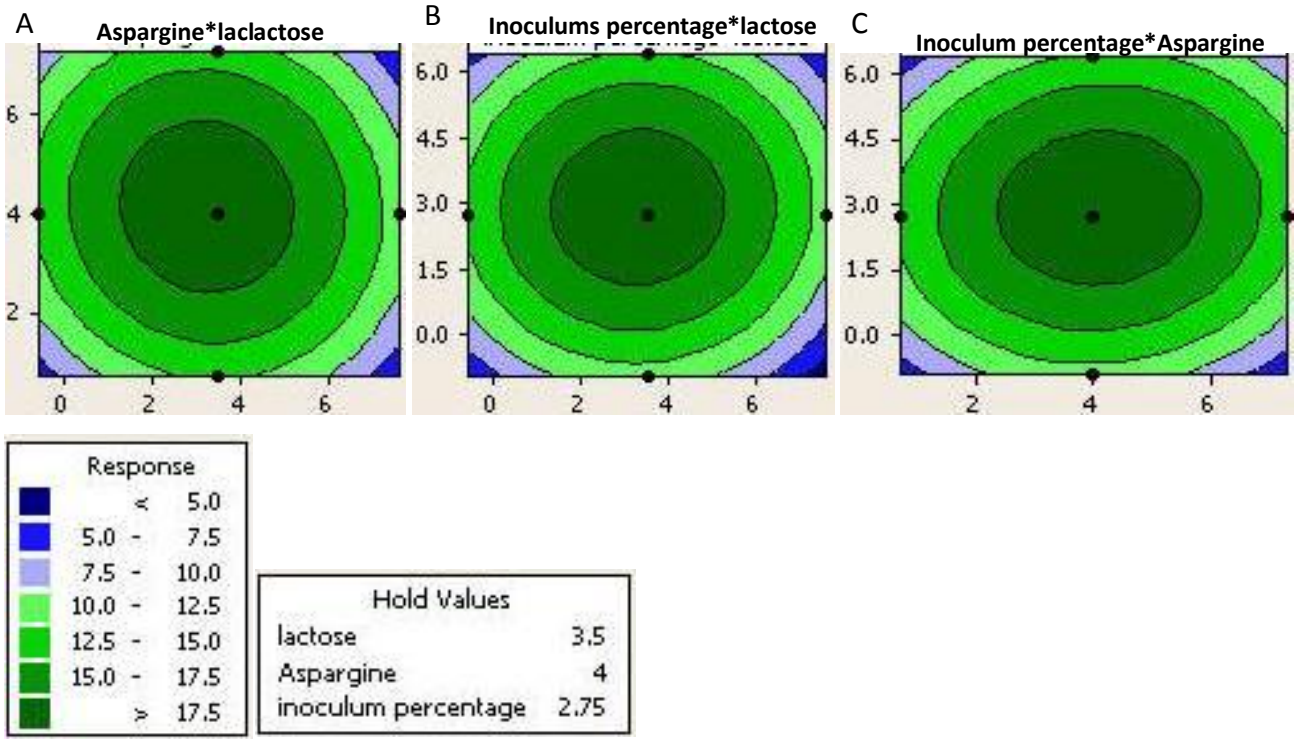

Figure 1. Two-dimensional contour plot for L -asparaginase production showing the interactive effects of (a) L-asparagine and lactose, (b) Lactose and inoculum size and (c) L-asparagine and inoculum size.

curvature was observed with lactose concentration and inoculum percentage showing an insignificant interaction. This non significant interaction was also confirmed by Student's *t*-test with *P*-value (*P* = 0.186) and *t*-value (1.431) as shown in Table 6. This result therefore corroborated the predicted values and the effectiveness of the model, indicating that the optimized medium favors the production of asparaginase.

The optimum levels of the variables were obtained by solving the regression equation and also by analyzing the response surface contour plots using Minitab software. The model predicted a maximum asparaginae activity of 19.33 U ml^{-1} appearing at lactose (3.5 g/L), asparagine (4.00 g/L) and inoculum percentage (2.75%).

Validation of the optimized condition

On the basis of medium optimization, the quadratic model predicted that the maximum production of asparaginase was 19.09 Uml^{-1}. To verify the predicted results, validation experiment was performed in triplicate tests. The RSM

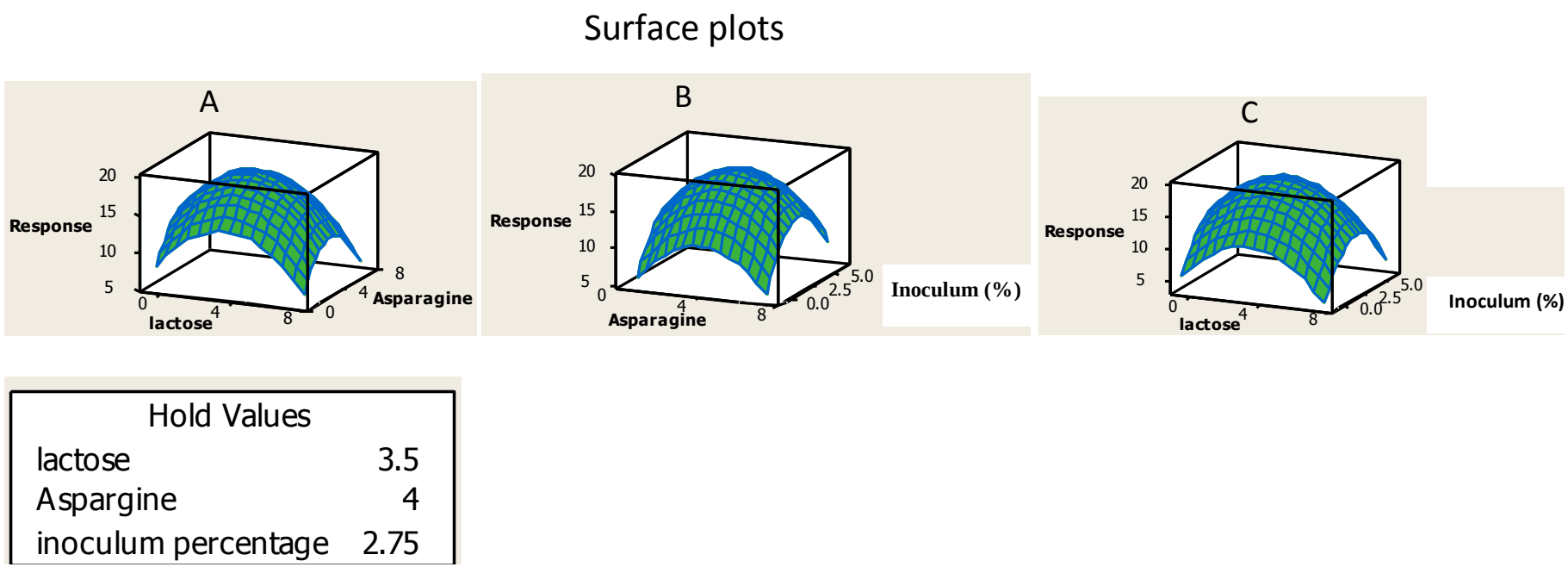

Figure 2. Three-dimensional response surface plot for L -asparaginase production showing the interactive effects of (a) L-asparagine and lactose, (b) L -asparagine and inoculum size and (c) lactose and inoculum size.

optimized data was validated by both shake flask and fermenter scale. The production of L-asparaginase was slightly higher (20.34 Uml^{-1}) in the fermenter than that in the shake flask at optimized conditions. Maximization for the production of L-asparaginase was achieved at 12 h in the fermenter, same as the case in shake flask and resulted in an increase in the enzyme activity. A significant increase in biomass production (from 0.396 to 0.632 g 50 ml) was also achieved in the fermenter. The results are presented in Table 7.

DISCUSSION

Nutritional requirements of microorganism play an important role during metabolite synthesis process. Among various nutritional requirements, carbon source and nitrogen source are generally regarded as important factors of metabolism, and several examples of the production of metabolites in media with optimized contents of these components are also cited in the literature. The statistical approaches have been successfully applied to improving media formulation for the production of primary and secondary metabolites in fermentation processes.

In general, glucose was regarded as a repressor for L-asparaginase. The production level of L-asparaginase was low in the basal medium containing glucose as reported previously (Barnes et al., 1977). In the present study, the production of L-asparaginase was enhanced in the presence of L-asparagine which is highly a specific substrate for L-asparaginase that reveals the inducing nature of this amino acid and bacterium can also metabolize the lactose for this growth associated production. However, there are recent investigations of medium development of L-asparaginase through this bacterium (Kumar et al., 2009a, b) but maximum enzyme activity obtained after using the statistically based experiments was 15.36 Uml^{-1}. This work demonstrates the efficacy of the combined methodology achieving a maximum productionof19.33Uml^{-1}insubmergedfermentation.

L-Asparaginases from *E. carotovora*, *Erwinia crysanthemi*, *E. coli*, and *S. cerevisiae* have been cloned and successfully expressed in bacterial and yeast expression systems (Khushoo et al., 2004, 2005; Kotzia and Labrou, 2005; Maria et al., 2006) and have been reported for many years as effective drugs in the treatment of acute lymphoblastic leuke-

Table 7. Comparison of L -asparaginase and biomass production in the initial and final optimized medium.

Type of medium	Enzyme activity (Uml^{-1})	Dry biomass (g 50 ml^{-1})
Unoptimized	01.20	0.014
RSM-optimized (shake flask)	19.33	0.496
RSM-optimized (fermenter)	20.34	0.632

mia. Their main side effects are anaphylaxis, pancreatitis, diabetes, leucopoenia, neurological seizures and coagulation abnormalities. Hence, an attempt has been made to find out novel sources of this enzyme which is accomplished through *P. carotovorum*. The present studies only envisaged the statistical approaches for media optimization for enhanced production of L-asparaginase through this bacterium. The glutaminase free asparaginase obtained from this bacterial source paved the way for future anti-cancer therapy.

The L-asparaginases of *P. carotovorum* in the future studies can be correlated into various directions: 1) the enzyme can be used for further screening as a potential anticancer agent *in-vivo*; 2) various protein designing techniques can be used for altering key enzyme properties including stability, product inhibition, enantioselectivity, function in non-natural environment; 3) development of biosensor for asparagine detection in leukemic patients.

Conclusion

This study proved that statistical experimental designs offer an efficient and feasible approach for asparaginase production. An enhanced enzyme activity was observed (19.36 U ml^{-1}) with the following optimized factors: lactose (3.5 g/L), asparagine (4.00 g/L) and inoculum percentage (2.75%). Validation experiments were also carried out to verify the adequacy and the accuracy of the model, and results show that the predicted value agreed with the experimental values well. The results also open the new avenues for the screening of potential anti-cancer agent *in vivo*.

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Full Length Research Paper

Influence of alcohol: oil molar ratio on the production of ethyl esters by enzymatic transesterification of canola oil

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The influence of alcohol:oil molar ratio on the canola oil transesterification reaction in solvent-free medium using free lipase from *Thermomyces lanuginosus* and *Burkholderia cepacia* was studied. The experiments conducted in batch reactor for 72 h at 37°C in cosolvent-free reaction system with ethanol addition in three steps showed great potential for ester production. The stepwise addition of ethanol allowed increasing yield throughout the total period of the reaction, even if the course has limited reaction at times, minimizing possible deleterious effects of the alcohol on the enzyme structure. The highest yields were achieved with lipase from *T. lanuginosus*, despite presenting lower activity values than those of *Burkholderia cepacia* lipase, which proved to be less selective for ester production. In the reaction medium containing lipase from *T. lanuginosus*, 100 % yield was obtained using a molar ratio of 12:1. For *B. cepacia* lipase, the highest yield was 90.73% at a molar ratio of 6:1. In all cases studied, at least 92% of the triacylglycerols from canola oil were consumed.

Key words: Free lipase, *Thermomyces lanuginosus, Burkholderia cepacia,* molar ratio, solvent-free medium, transesterification reaction, triacylglycerols, diacylglycerols, monoacylglycerols, ester.

INTRODUCTION

A special kind of enzymes that can be used in the production of esters are the lipases, which are present in living organisms and are used for the hydrolysis and synthesis of triacylglycerols. The enzymatic route is preferred over the chemical route due to the environmental appeals of green chemistry, the possibility of esterifying oils with high fatty acids content, and also the feasibility of conducting the reaction under mild conditions of temperature and pH. In most ester production processes in the world, the alcohol used is methanol, as it is more reactive and reduces emulsification problems, that is, facilitates product purification. However, ethanol

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Abbreviations: HPLC, High performace liquid chromatography; **A,** ultra-pure water; **B,** acetonitrile; **C,** 5:4 (v/v) isopropanol:hexane; **TAG,** triacylglycerols; **DAG,** diacylglycerols; **MAG,** monoacylglycerols.

is more interesting from the viewpoint of technological sustainability, as long as it comes from renewable sources, while methanol is usually a petroleum derivative. In addition, ethanol is produced in large scale in Brazil.

In reactions that use inorganic catalysts, excess alcohol is used to ensure high conversion and minimize diffusional restrictions.

In enzymatic synthesis, however, excessive levels of alcohol can inhibit the enzyme and decrease its catalytic activity during the reaction. According to the study of Salis et al. (2005) higher alcohol:substrate ratio means higher polarity of the medium that may be associated with the inactivation of the biocatalyst or even the possibility of destabilizing the essential water layer of its catalytic site (Köse et al., 2002). In order to minimize the effects of enzyme deactivation by excess alcohol, Watanabe et al. (2001) addition into the reaction medium was performed stepwise. Given the above, this work was planned to investigate the influence of ethanol in the reaction medium on ethyl esters production by transesterification of canola oil in solvent-free medium, using free lipases from *Thermomyces lanuginosus* and *Burkholderia cepacia*.

MATERIALS AND METHODS

Reagents

Amano P.S. lipases from *B. cepacia* and *T. lanuginosus*, 2-phenethyl alcohol, vinyl acetate, 2-phenethyl acetate, and diisopropyl ether were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. Absolute ethanol 99.9% was from Cinética, Brazil. Commercial canola refined oil was purchased in a local supermarket. The reagents used in high performance liquid chromatography (HPLC) were n-hexane, n-propyl alcohol and acetonitrile (J.T. Baker). The standards tri-olein, di-olein, mono-olein and methyl-oleate were purchased from Accustandard. Other materials and reagents were of analytical grade.

Methods

Transesterification reaction

The reactions of ethyl esters synthesis were performed in a jacketed cylindrical batch reactor with a capacity of 50 mL of reaction medium, which contained the oil used in the experiment, ethanol at alcohol:oil molar ratios of 3:1, 6:1, 9:1, and 12:1, and free lipase (5 % of the oil mass). The reaction was performed for 72 h at 37°C at 250 rpm. Ethanol was added in three steps (equal amounts at the beginning, after 12 and 24 h).

The reaction sample were washed with hot water to inactivate enzyme, centrifuged, dried and storage at -20°C. Production of ethyl esters and consumption of triacylglycerols were monitored by HPLC.

Determination of transesterification activity by 2-phenethyl acetate production

Transesterification activity in the reaction of 2-phenethyl acetate formation using 2-phenethyl alcohol and vinyl acetate was measured by high performance liquid chromatography (HPLC, Varian 920-LC) with ODS-C18 column and UV-VIS detector at 254 nm, using acetonitrile and water (42:58) as mobile phase, room temperature, injection of 10 µL, and flow rate of 1.0 $mL.min^{-1}$. The reaction mixture was prepared by adding 0.6 mL of 2-phenethyl alcohol and 2.4 mL of vinyl acetate to 20 mg of the biocatalyst and kept under stirring for 20 min at 37°C at 200 rpm. The method of transesterification activity by 2-phenethyl acetate production was proposed by the supplier Amano P.S. as assay method of transesterification activity.

Analytical method

A combined linear gradient with aqueous/organic and non-aqueous phases, adapted from Holčapek et al. (1999) was used for HPLC. The total time of the gradient was 42 min, using ultra-pure water (A), acetonitrile (B), and 5:4 (v/v) iso-propanol:hexane (C). The method started with 70% A + 30% B, going to 100% A in 15 min and then to 50% A + 50% C in 11 min, followed by 10 min of isocratic elution with 50% A + 50% C.

Over the last 6 min the initial equilibrium condition was restored (70% A + 30% B). The column was operated at 40°C with UV-VIS detection at 205 nm, with injection of 20 µL and flow rate of 1 $mL.min^{-1}$. Quantification of triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG), and ester was carried out by the sum of peak areas, with subsequent application in the calibration curves obtained with external standards. Triacylclycerol consumption and yield of product formation were determined by Equations 1 and 2, respectively,

$$C_T = \frac{\text{moles of TAG consumption}}{\text{moles of TAG initial}} \quad (1)$$

$$Y_i = \frac{\text{moles of product i formed}}{\text{moles of product i that can be formed}} \quad (2)$$

Where, C_T, is the Triacylclycerol consumption; Y_i, is the yield of product formation.

Ester production activity and triacylglycerol consumption activity were determined from the initial velocities of ester production and triacylglycerol consumption. A unit of activity was defined as the amount of enzyme required for producing 1 µmol of product per minute.

RESULTS AND DISCUSSION

Transesterification activity

It is difficult to characterize lipase enzymes activities since most enzymes carry out only one specific reaction (Babtie et al., 2010). Thus, the activity can be measured and compared by means of a single standard reaction. Ordinarily, lipases are promiscuous enzymes (Kapoor and Gupta, 2012), in other words, can catalyze both the hydrolysis of triacylglycerols and their formation. Thus it is difficult to characterize and compare the potential activity of lipases and prevents further discuss phenomena involved or compare lipases of different papers. Given

Table 1. Triacylglycerol consumption activity (A_{TC}), ethyl esters production activity (A_{EP}), consumption of TAG (C_T), and esters yield (Y_E).

Alcohol:oil molar ratio	*Thermomyces lanuginosus* lipase				*Burkholderia cepacia* lipase			
	A_{TC} (U/g)	A_{EP} (U/g)	C_T (%)	Y_E (%)	A_{TC} (U/g)	A_{EP} (U/g)	C_T (%)	Y_E (%)
3:1	9.44	22.15	100	86.81	36.50	106.70	95.07	60.78
6:1	8.52	22.42	100	99.94	22.50	20.04	100	53.28
9:1	11.79	59.62	100	97.38	30.54	110.46	95.64	67.26
12:1	3.49	20.14	100	97.66	18.88	19.01	95.95	54.54

this difficulty, Frédéric et al. (2000) reviewed a large number of methods to measure the hydrolytic activity of lipases, Sörensen et al. (2010) quantifies the activity by the method of 4-nitrophenyl acetate as well as Dhake et al. (2013) even though the methods used were different. Given the difficulty in characterizing, Chen et al. (2012) using two methods to quantify the activity of the immobilized lipase activity is a method of hydrolysis of olive oil and the other is the esterification of decanoic acid. Rodríguez-Contreras et al. (2012) also uses the method of hydrolytic activity equivalent to that used by Chen et al. (2012). In this work, lipase activity is measured by transesterification activity. Thus we can represent classes of reactions that are involved in this work.

The transesterification activity per unit mass of protein in the reaction of formation of 2-phenethyl acetate using 2-phenethyl alcohol and vinyl acetate were 73.93 and 284.16 U·g-1 for *T. lanuginosus* and *B. cepacia* lipases, respectively. Additionally, we chose to also discuss triacylglycerol consumption activity (A_{TC}) and ethyl esters production activity (A_{EP}), which is described in Table 1. Also, we construct an extensive discussion on the values of the enzyme in the reaction compared to the values of yield ester and triacylglycerol consumption.

Optimization of alcohol:oil molar ratio

The different behaviors shown by lipases from *T. lanuginosus* and *B. cepacia* can be observed in Figures 1 and 2, respectively, with variation of the amount of alcohol in the reaction medium. Figure 1A shows a rapid consumption of TAG up to 120 min, when there is a reduction in the consumption of these compounds. It is also possible to observe that the yield of esters and DAG grows markedly in the same period and then the reaction slows down, indicating that the stepwise addition of alcohol caused limitation of this reagent in the reaction medium, leading to a decrease in reaction rate. After 720 min of reaction, a new portion of ethanol was added and then the reaction resumed, but at lower speed. At the end of the tests, 86.81% ester yield was achieved, with complete consumption of TAG and only DAG (12.38%) and MAG (8.45%) remaining in the reaction medium. The same behavior is repeated in Figure 1B, but for the alcohol:oil molar ratio of 6:1 the decrease in reaction rate occurred after 240 min. It is important to note that even with low alcohol levels, between 240 and 720 min, discrete consumption of DAG forming MAG and esters was still observed. After the second addition of alcohol at 720 min, all reaction intermediates were rapidly consumed, reaching 99.94% ester yield, with little amounts of MAG and no DAG or TAG. Small amounts of TAG, DAG and MAG is important for application of esters as biodiesel, because these compounds must be removed in the purification step.

In Figure 1C, the reduction of both ester production and triacylglycerol consumption rates occurred at 360 min, but the rate of DAG production was exceeded by the rate of consumption after around 120 min. In this configuration, with alcohol:oil molar ratio of 9:1, no lack of alcohol was expected in the reaction medium, as the amount of alcohol added in the first step was enough for the reaction to be completed. That was not the case, though, as between 360 and 600 min the reaction showed a decrease in the rate of consumption of reagents. After the addition of more alcohol, triacylglycerol and diacylglycerol consumption was resumed and low amounts of intermediates were found after 1440 min of reaction. At the end of the experiment 97.38% of the esters had been formed. As for alcohol:oil molar ratio of 12:1 (Figure 1D), no decrease in reaction rate was observed and 360 min of reaction led to 99% of TAG consumption and 85.95% of ester yield. After 480 min, TAG were depleted and 97.97% of the esters had been formed. It is clear from Figures 1A, 1B, and 1C that within 12 h of reaction there was a lack of ethanol in the reaction medium, even with alcohol:oil molar ratio of 9:1 in which the minimum amount of alcohol was added early in the reaction. This indicates that the alcohol is not only a reactant of the reaction medium, but also plays the role of the solvent reaction medium. Indeed Avelar et al. (2013) which verified a yield reduction on the increasing of concentration of the oil in the reaction medium. This effect was attributed to aggregation of the oil droplets that eventually resulted in destabilization of the emulsion.

However, the lack of ethanol in the reaction medium did

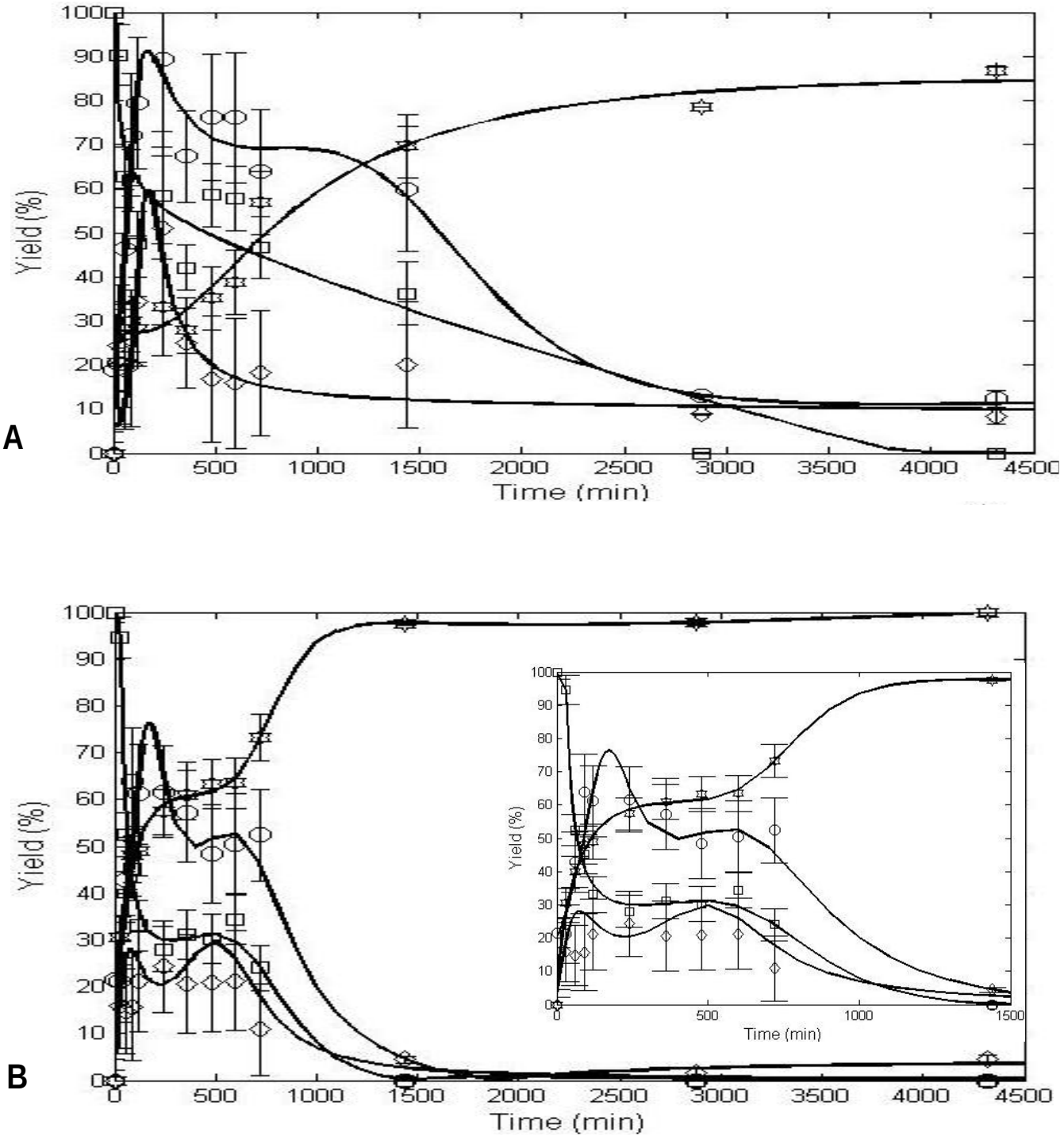
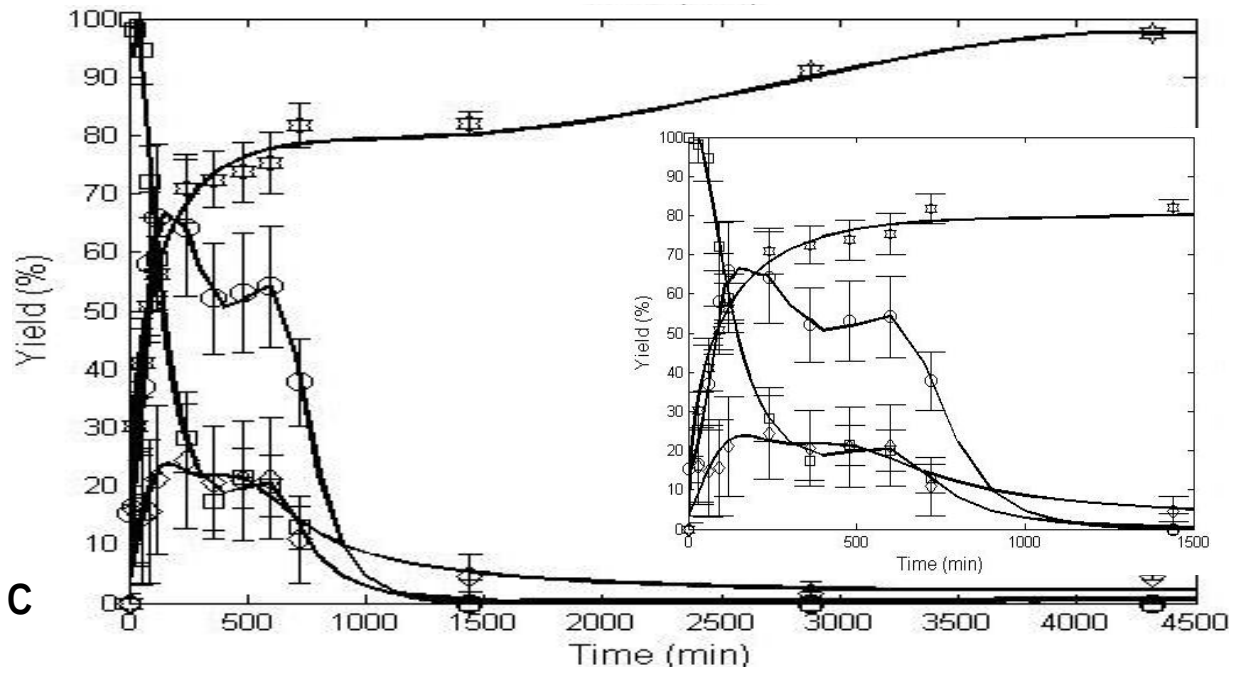


Figure 1. Yield profile of the transesterification reaction products using lipase from *Thermomyces lanuginosus*: Triacylglycerol (□), diacylglycerol (Δ), monoacylglycerol (○), and ethyl esters (◌). Alcohol:oil molar ratio: 3:1 (A), 6:1 (B), 9:1 (C), and 12:1 (D).

not affect the reaction yield because when new ethanol aliquots were added, all reactions proceeded. Figures 3 and 5 show that even after the second addition of alcohol the reactions with lower amounts of ethanol proceeded more slowly. It is also interesting to note in Figure 1 that the concentration of MAG was always smaller than that of DAG, indicating that the slow step of the reaction was the transesterification of DAG to esters and MAG. As a



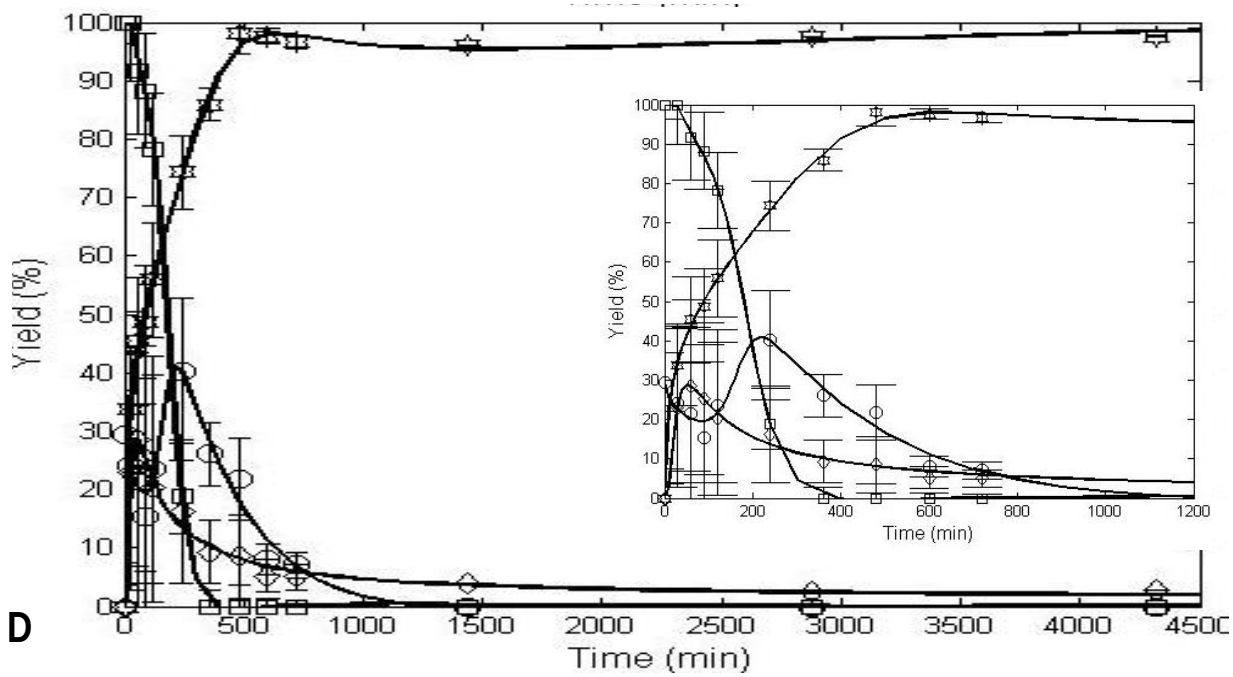


Figure 1. Contd.

matter of fact, Shu et al. (2011) studying the kinetics of biodiesel production using a solid acid catalyst, found that transesterification is the slowest step in the reaction of ester production. The results for lipase from *T. lanuginosus* show that high alcohol:oil molar ratios provide better yields, and then high amounts of ethanol

rapidly promotes the reaction. However, ethanol should be used cautiously. Excess alcohol can cause deformation of the essential water layer that stabilizes the enzyme active site, or destabilization of the alcohol/oil interface where the catalytic action of the enzyme takes place (2, 4, 16). However, this was not observed in the results, since the production of esters increased throughout the test period for all analyzed conditions.

Verdugo et al. (2011) investigating the production of ethyl esters from sunflower oil with high fatty acid content using lipase from *T. lanuginosus* as catalyst obtained maximum ester yield of 70%. Studying biodiesel production from canola oil and methanol, Dizge et al. (2009) used *T. lanuginosus* lipase immobilized as catalyst obtained yield of 97% in 24 h batches at 50°C, with stepwise addition of alcohol, using molar ratio of 6:1. Dizge and Keskinler (2008) obtained a maximum yield of methyl ester of 90% at 40°C with alcohol:oil molar ratio of 6:1 and adding 0.1 g of water into the reaction medium. Comparing the results of the present study with those reported by the authors mentioned above, it is clear that the best ester yield (greater than 99%) was found with alcohol:oil molar ratio of 6:1. The results were consistent with those obtained by the aforementioned authors even with the use of free enzyme in the reaction medium, considering that the results were obtained at 37°C and after 72 h of reaction.

Ethyl ester production from *B. cepacia* is described in Figure 2. Fast consumption of TAG can be observed up to 720 min in Figure 2A. After that, reaction progress became very slow and by the end of the experiment, triacylglycerol consumption was 95%. Ester yield profile showed a very similar behavior, with fast production up to 31.84% yield at 360 min, followed by a slow production period which led to the maximum yield of 60.78% after 4320 min. Diacylglycerol production increased rapidly up to 240 min (60%) and decreased to 22.74% between 480 and 720 min. By the end of the experiment its value was 13.88%. MAG showed maximum yield of 10.08%. Figure 2B shows the rapid consumption of TAG between 30 and 720 min. After 2880 min of reaction they had been completely converted. Ester yield showed a sharp growth up to 480 min, followed by a slow evolution until reach 53.28% yield. The maximum yield of DAG was 48.37% after 600 min, and by the end of the experiment, its value was 10.53%. MAG showed a maximum yield of 23.67% and at the end of the reaction the yield was 19.61%. Figure 2D showed the highest yields of MAG, when compared with the other molar ratios, reaching a maximum yield of 33.48% after 1440 min and 20% at the end of the reaction. The maximum yield of DAG was of 43.96% at 480 min. TAG were rapidly consumed up to 1440 min, reaching 93%, and then remained constant until the end of the reaction. The highest yield of esters was found to be 67.26%. Analysis of Figure 2 shows that in all cases DAG yield profiles were higher than those of MAG, indicating that the slow step in the reaction was the conversion of DAG to MAG and ethyl esters, unlike what happens for the reaction using *T. lanuginosus*. All alcohol:oil molar ratios tested with lipase from *B. cepacia* showed equivalent ester yields, except the molar ratio 9:1, whose yield of 67% was slightly higher than the others. Alcohol:oil molar ratio also had little influence on the consumption of TAG; 100 and 96% were consumed for 6:1 and 12:1 molar ratios, respectively.

Studying a new strain of *B. cepacia*, Yang et al. (2007) conducted the transesterification of soybean oil at 40°C with methanol:oil molar ratio 3:1, 5% water, and 3% enzyme. The authors obtained up to 88% ester yield after 72 h of reaction. Da Ros et al. (2010) evaluated the catalytic properties of *B. cepacia* lipase immobilized for the synthesis of biodiesel at 50°C for 48 h with ethanol:oil molar ratio of 12:1 and using 20% of immobilized enzyme. The best ester yields obtained with babassu oil and tallow were 100 and 90%, respectively. Salum et al. (2010) produced biodiesel in fixed bed reactor with lipase from *B. cepacia* LTEB11 and obtained ester yield of 40% from soybean oil after 48 h of reaction at 37°C with alcohol:oil molar ratio of 6:1, and using n-heptane as cosolvent.

Ester yields obtained in the present work were lower than those reported by the above cited authors. However, the present results were obtained with no addition of cosolvent to the reaction medium. Lipase may be inhibited in the reaction of ethyl esters production by transesterification of TAG from oils when there is no addition of cosolvents (Royon et al., 2007; Véras et al., 2011; Hama et al., 2007; 2009; Fernandes et al., 2007). This inhibition occurs as short-chain alcohols and glycerol are capable of forming a hydrophilic coat on the surface of the enzyme that excludes the triacylglyceride from the active site (Véras et al., 2011; Hama et al., 2007; Adachi et al., 2011; Tan et al., 2010). The cosolvent is believed to reduce the interfacial tension that arises in solution, mainly due to increase of triacylglycerol and ethanol solubility (Véras et al., 2011; Tan et al., 2010). Faster reactions are then possible due to the higher stability of the active site.

Figures 3 and 4 show the rapid consumption of TAG up to 360 min for *T. lanuginosus* lipase and up to 720 min for *B. cepacia* lipase, which is quantified by the triacylglycerol consumption activity (A_{TC}) shown in Table 1. *B. cepacia* lipase was more efficient than that from *T. lanuginosus* in the conversion of TAG, as it presented higher values of A_{TC}. Table 1 also shows triacylglycerol consumption (C_T) for both enzymes. It is interesting to note that in spite of showing higher A_{TC} values, *B. cepacia* lipase presented lower C_T values than that from *T. lanuginosus*, except for the 6:1 molar ratio, in which the consumption was the same for both enzymes. Low activity values indicate hindering of the reaction progress by a deleterious effect on the structure of the enzyme.

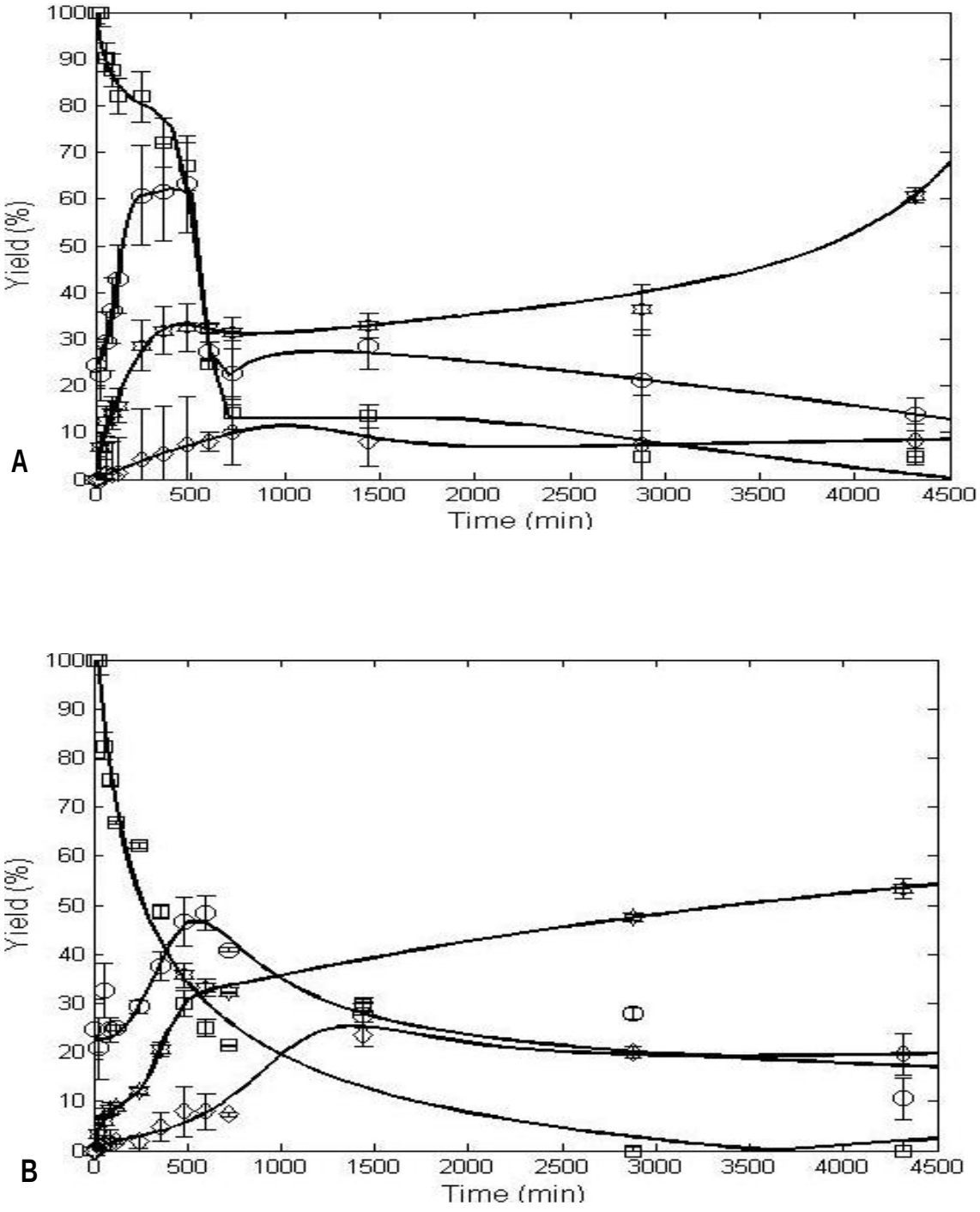


Figure 2. Yield profile of the transesterification reaction products using lipase from *Burkholderia cepacia*: Triacylglycerol (□), diacylglycerol (Δ), monoacylglycerol (○), and ethyl esters (۞).. Alcohol:oil molar ratio: 3:1 (A), 6:1 (B), 9:1 (C), and 12:1 (D).

This would result in a slower reaction, but the opposite behavior was observed; higher levels of triacylglycerol consumption were reached by the enzyme that showed lower activity. This suggests that the reaction medium inhibits the lipase from *B. cepacia* and not that from *T. lanuginosus*.

Figures 5 and 6 show ester yield progress during the reaction. There was stagnation in production for lipase

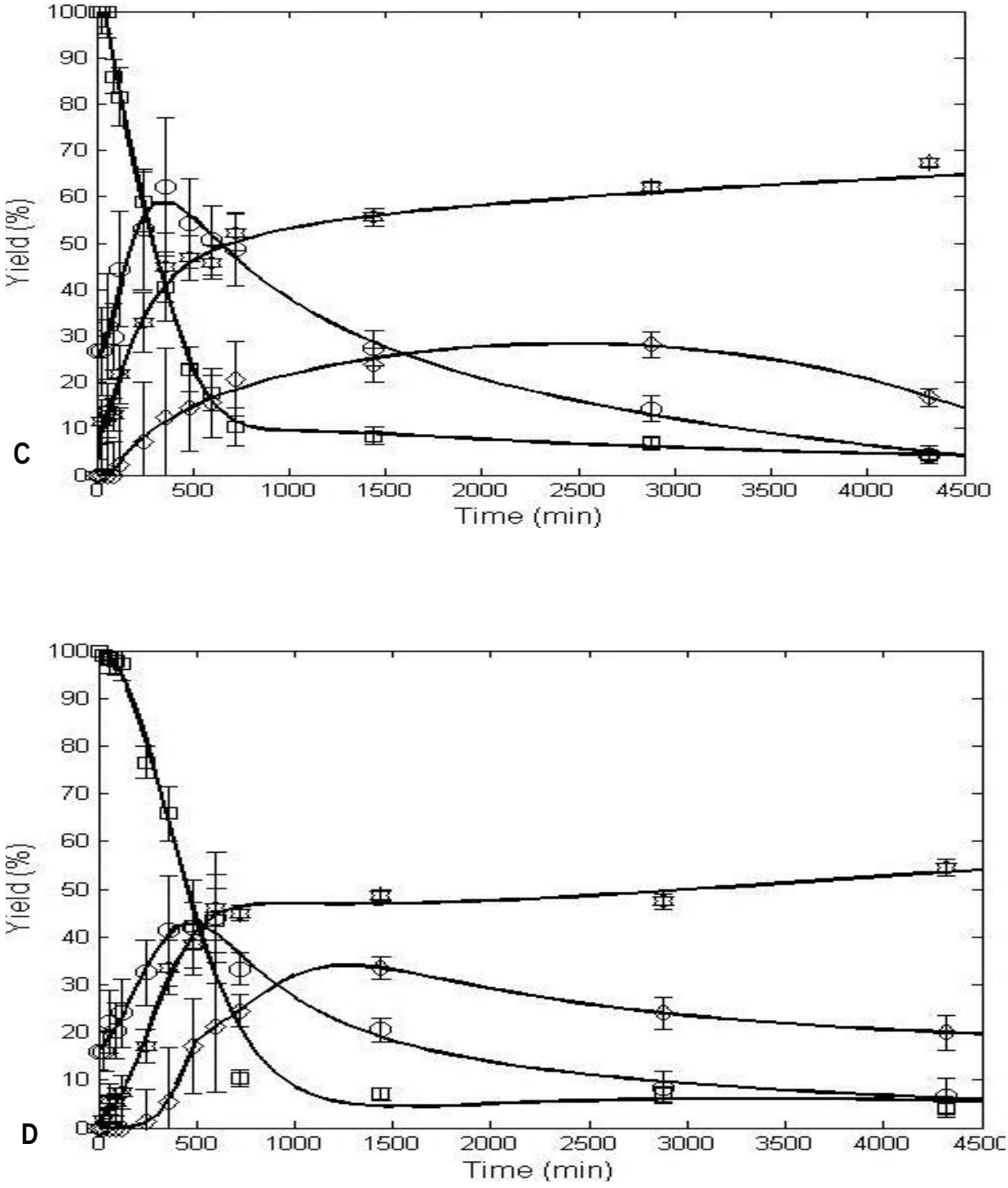


Figure 2. Contd.

from *T. lanuginosus*, probably due to lack of alcohol in the reaction medium because of the stepwise addition of this reagent. After addition of the second ethanol aliquot, the reaction proceeded normally, but with a decrease in reaction speed, except for the molar ratio of 12:1, in which the effect of rate reducing was not observed. When lipase from *B. cepacia* was used, no stagnation or reduction in reaction speed was observed. Interestingly,

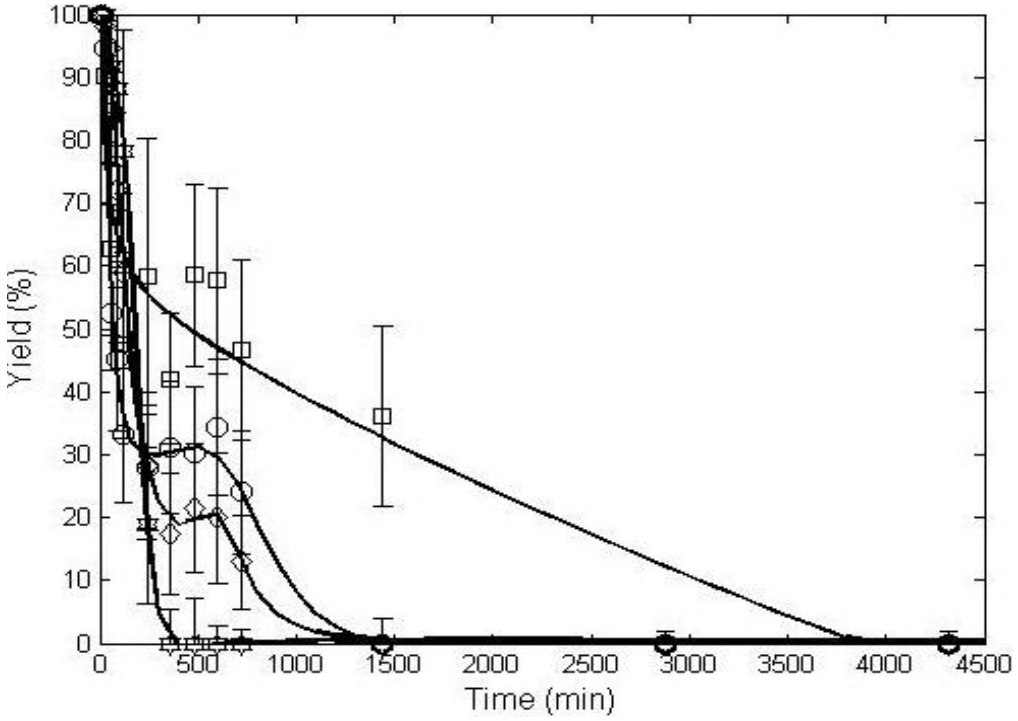


Figure 3. Consumption of triacylglycerols by *Thermomyces lanuginosus* lipase. Ethanol:oil molar ratio 3:1 (□), 6:1 (○), 9:1 (◊), and 12:1 (۞).

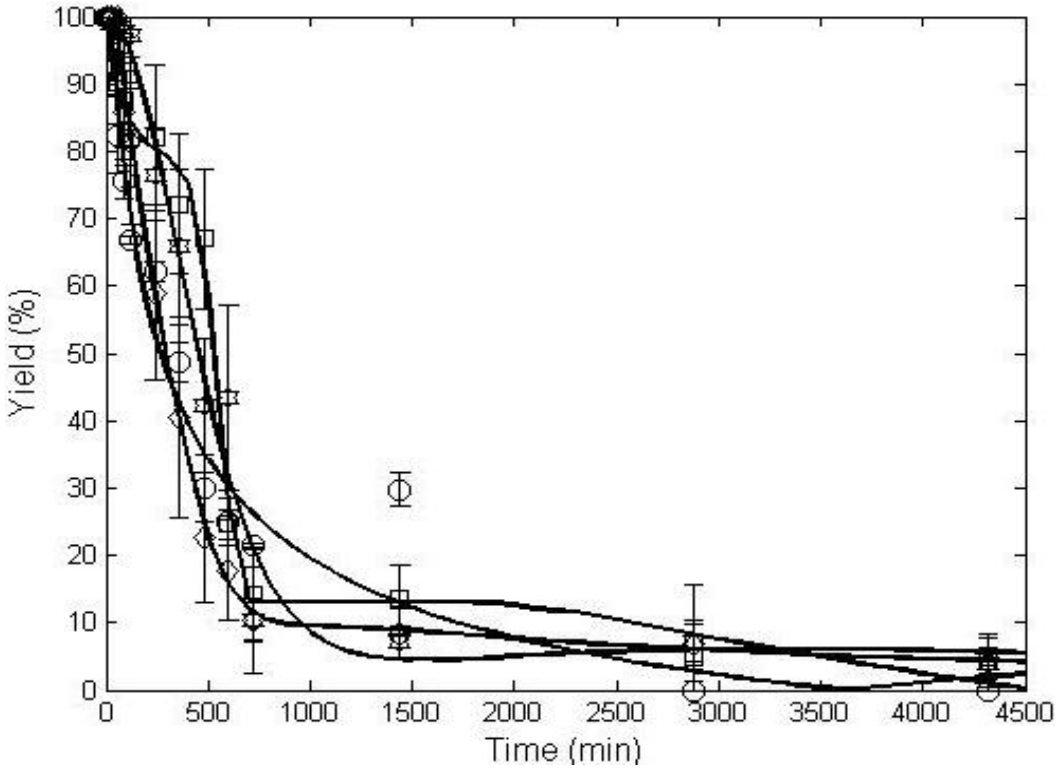


Figure 4. Consumption of triacylglycerols by *Burkholderia cepacia* lipase. Ethanol:oil molar ratio 3:1 (□), 6:1 (○), 9:1 (◊), and 12:1 (۞).

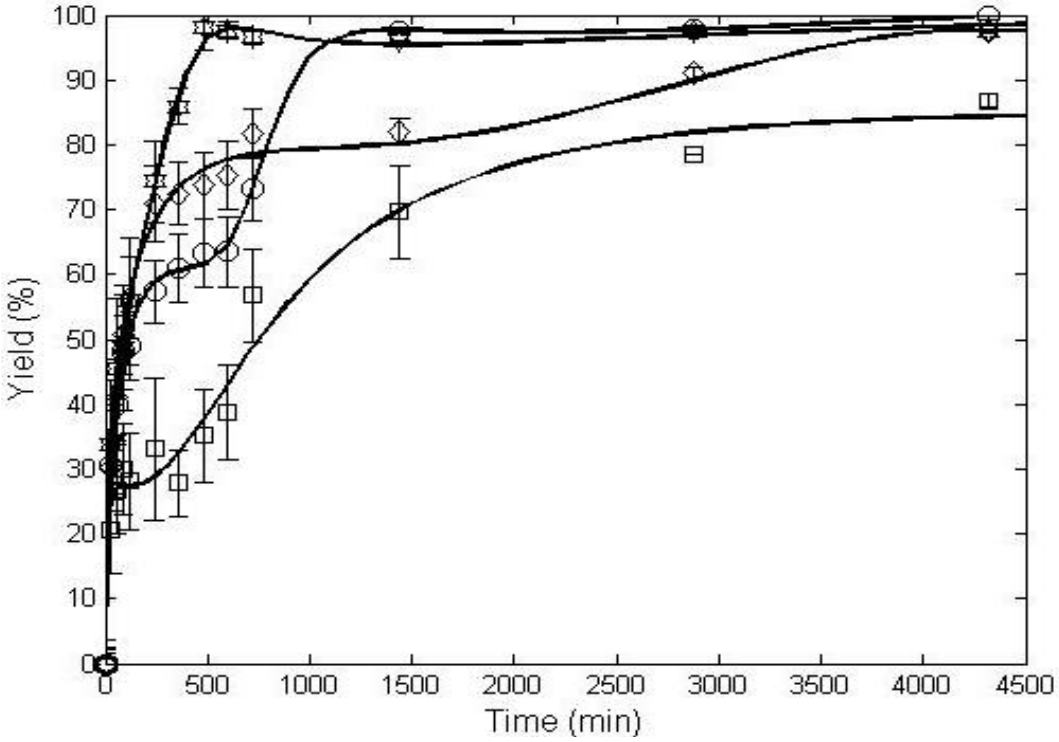


Figure 5. Ethyl esters production by *Thermomyces lanuginosus* lipase. Ethanol:oil molar ratio 3:1 (□), 6:1 (○), 9:1 (◊), and 12:1 (۞).

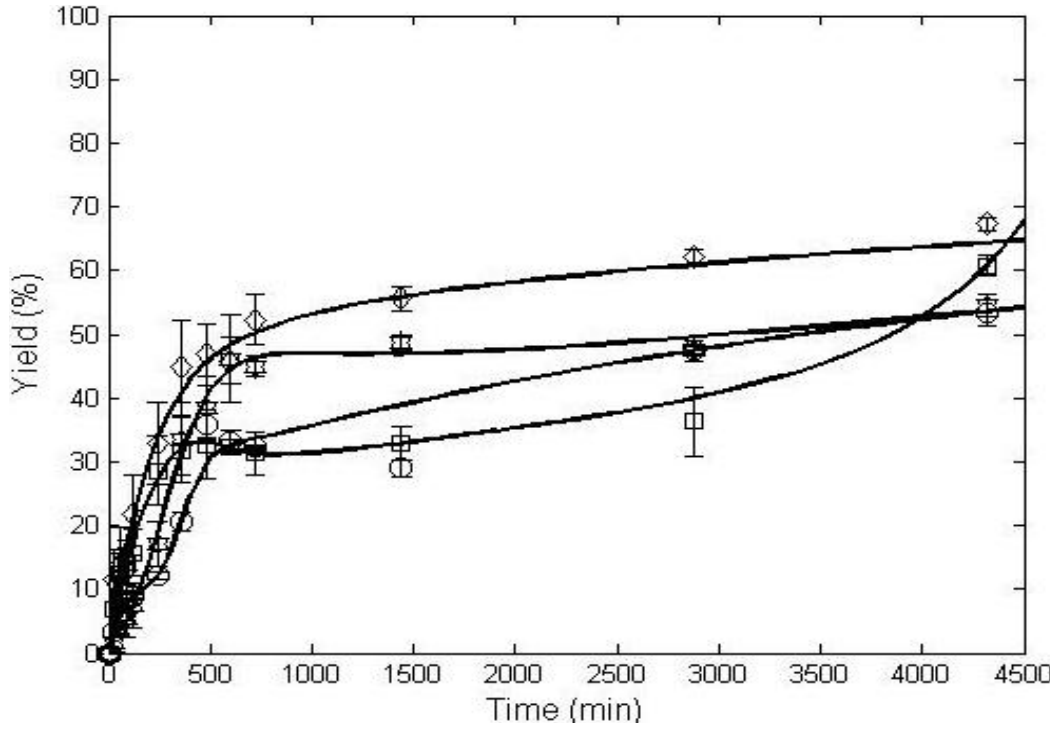


Figure 6. Ethyl esters production by *Burkholderia cepacia* lipase. Ethanol:oil molar ratio 3:1 (□), 6:1 (○), 9:1 (◊), and 12:1 (۞).

at alcohol:oil molar ratio of 9:1 with *B. cepacia* lipase, no lack of alcohol was observed in the reaction medium, as the minimum amount of alcohol required had been added in the first aliquot. On the other hand, this lack of alcohol was observed when using *T. lanuginosus* lipase, as shown in Figure 5, with a decrease in the rate of production of esters at around 500 min. This rate reduction suggests that the enzymes present different mechanisms of action, which is confirmed by the finding that the slow step in the reaction was the conversion of DAG to MAG and ethyl esters, unlike what happens for the reaction using *T. lanuginosus*. The effect of reaction rate reduction with *T. lanuginosus* lipase is also evident in Table 1, where higher ester production activity is observed for *B. cepacia* lipase than for *T. lanuginosus* lipase, except for alcohol:oil molar ratios of 12:1 and 6:1, which shows equivalent activities. Despite presenting lower activity values, *T. lanuginosus* lipase showed higher ester yields than *B. cepacia* lipase, as already observed for triacylglycerol consumption.

In a 2^2 factorial design experiment using *Burkholderia cepacia* lipase, Fernandes et al. (2007) observed little influence of alcohol:oil ratio in the reaction medium on the production of ethyl esters of corn oil at 37°C with the use of cosolvent. The yield changed very little with a twofold increase in the molar ratio. These results corroborate the indication that for *B. cepacia* lipase an increase in alcohol:oil molar ratio not necessarily leads to a significant increase in ester yield.

Conclusions

Lipase from *B. cepacia* was less selective for ethyl esters production than that from *T. lanuginosus*. The differences in efficiency of esters production may be directly related to the different reaction mechanisms of each enzyme. The slow step of the transesterification reaction was the conversion of monoacylglycerol to ester for *T. lanuginosus* lipase and the production of ethyl esters and monoacylglycerol from diacylglycerols for *B. cepacia* lipase despite the stepwise addition of ethanol to the reaction medium in order to minimize the effects of enzyme deactivation. The lower activity for ester production by *T. lanuginosus* lipase when compared with *B. cepacia* did not impair the progress of the reaction, with 99.94% ester yield for alcohol:oil ratio of 6:1. The highest yield achieved with lipase from *B. cepacia* was 67.26%, with molar ratio of 9:1. The conversion of triacylglycerols in the reaction medium with lipase from *T. lanuginosus* was complete in all conditions, while for the reaction medium using lipase from *B. cepacia* 100% conversion was achieved only with alcohol:oil molar ratio of 6:1. The results indicated the potential of lipase to produce esters in cosolvent-free media.

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Full Length Research Paper

Evaluation of anti-hyperglycemic activity and side effects of *Erythraea centaurium* (L.) Pers. in rats

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In folk medicine, several plants, among which the small centaury, are recommended for the treatment of diabetes type 2 in humans. An experimental study to evaluate anti hyperglycemic effect of *Erythraea centaurium* (L.) Pers. was performed on wistar rats. Normoglycemic rats and rats subjected to oral glucose tolerance test overload "OGTT" were used. Administration to these animals by gavage of 20% aqueous extracts (at a dose of 0.66 ml/100 g body weight) and butanolic extract (at a dose of 0.015 ml/100 g body weight) of *E. centaurium* allowed to note, after the kinetic study of glucose between t0 and t180 min, a significant reduction in blood glucose levels. The anti hyperglycemic action of butanolic and aqueous extracts was compared to that obtained by the administration of Glibenclamide "Daonil® 5mg (ND)" at a dose of 0.25 mg/100 g weight body as a reference drug. This study showed the anti hyperglycemic property of the small centaury. But in the medium term, the administration of this plant in rats showed adverse effects on the liver and kidney.

Key words: *Erythraea centaurium*, aqueous extract, butanolic extract, anti-hyperglycemic effect, rats.

INTRODUCTION

Diabetes mellitus is one of the major diseases currently affecting the citizens of both developed and developing countries. It is estimated that 143 million people world-wide are affected by this disease and the number is growing rapidly (Maiti et al., 2004; Mentreddy et al., 2005). It is the fourth leading causes of death in the most developing countries (Arumugam et al., 2013). Some of the major reasons for the increasing rate of Type 2 diabetes also called non-insulin dependent diabetes are stress, and lack of proper diet and physical exercise (Mentreddy et al., 2005). The therapies currently available for diabetes include insulin and various oral antidiabetic agents such as sulfonylureas, biguanides, a-glucosidase inhibitors, and glinides, which are used as monotherapy or in combination. Many of these oral antidiabetic agents have a number of serious adverse effects. Therefore, the search for more effective and safer hypoglycemic agents has continued to be an important area of investigation (Jung et al., 2006). Plant-based medicinal products have been known since ancient times. The ethno botanical studies report about 800 plant species that may possess anti diabetic properties. Several plant species have been used for prevention or managing diabetes by the Native Americans, Chinese, South Americans and Asian Indians (Alarcon-Aguilara et al., 1998; Mentreddy et al., 2005). Traditional medicine is

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used for treatment of diabetes in developing countries where the cost of conventional medicines is a burden to the population (Saravanan and Pari, 2008). This study investigates the anti hyperglycemic activity of small centaury on rats.

The action of this herb was also compared with a sulfonyl urea, glibenclamide (Daonil ®) as a reference drug. The choice of the small centaury is based on surveys of the population and herbalists' which use this plant in some regions of Algerian country. It has been reported that the whole herb of centaury is appetite-stimulating, aromatic, bitter, cholagogic, diaphoretic, digestive, emetic, weakly febrifugal, hepatic, stomachic and tonic (Nada Mijajlović et al., 2005), hhypoglycemic, antipyretic, cardio-regulator, depurative, cicatrizing and hair-care (Bellakhdar et al., 1991). The chemical composition of the essential oil of this plant was investigated by Jerković et al. (2012).

MATERIALS AND METHODS

Plant materials

Plant identified by a specialist (Ms Khalfallah, Faculty of Science, University of Mentouri Constantine) was harvested in April in the region of Jijel and a specimen was preserved in our herbarium. The aerial parts of the plant were dried in the shade at room temperature for a period of 7 days. From the aerial parts of the plant, 4 dried extracts were obtained: dry petroleum ether, dry dichloromethane, dry ethyl acetate and 1-butanol.

Animals

Wistar adults' rats from the two sexes, with an average weight of 200 to 360 g were used. They were kept in the animal housed in cages under standard laboratory conditions and fed rabbits pellets and watered *ad libitum* until the end of the experiment.

Materials and chemicals

Balance device for restraining rats, metal cannula for feeding timer, 20 µl Eppendorf tubes containing heparin, tubes (for serum), pipettes tips (0.5A 5 ml, of 10 µl and 1 ml), bath (Grant brand temperature 20 to 90°C), glucometer (Roche) + strips, P Selecta centrifuge, precision balance (Precisa 3100C), Spatula, resistance (Stuart SB162 Series) were used for the study.

Chemicals

50% glucose solution, saline, aqueous extract, 20% butanol extract, glibenclamide (5 mg Daonil®), tranquilizers (acepromazine), anesthetics (xylocaine 2% non adrenaline) were purchased from Faculty of Science.

Experimental protocol

Rats were randomly divided in 5 groups of 4 rats each. Each rat was weighed and marked a significant sign. The day of the experiment, rats were fasted for 12 h. The different groups are the following: group 1: normal rats (NC); group 2: hyperglycemic untreated (HG); group 3: hyperglycemic treated with aqueous extract (HG + AE); group 4: hyperglycemic treated with butanol extract (HG + BE). Animals were gaved at t0 for group 1 and 2, at t60 min for groups 3 and 4, and at t5 mn for the 5th group. The blood samples were taken at t0, t60 min, t90 min, t120 and t180.

Drugs administration

Rats were overfed by plant extracts at t0 (60 min before the glucose overload); each of the animals in the (HG + AE) and (HG + BE) received the plant extract at a dose of 0.66 ml/100 g body weight and 0.015 ml/100 g body weight, respectively. Taking into account the daily amounts recommended by traditional healers for man, an aqueous extract at 20% w/v was prepared with the aerial parts of the plant (flowers, leaves and stem). The butanol extract was diluted in 1.5 ml of saline. At t60 min, each of the rats in the control group received 1 ml of absolute physiological saline (sodium chloride NaCl 9%). All other rats were given with other batches of the solution 50% glucose in distilled water (which corresponds to 0.8 ml/100 g body weight). The test was performed as described elsewhere (Alaoui et al., 1995; Hmamouchi et al., 1995). The last batch was received just before the glucose solution; the-glibenclamide was applied at standard-dose of 0.25 mg/100 g body weight (1.5 ml diluted in distilled water). The blood samples were made in caudal vein by section of the bright end of the tail, saphenous vein, after calming (acepromazine) and local anesthesia (xylocaine at 2% non adrenaline) and finally by cardiac puncture after anesthesia (just before sacrification animals).

Glycemic determination

The equipments used for the quantitative determination of glucose were the glucometer and spectrophotometer. For the 1st method, the blood glucose test-method was used directly as described by Andrade-Cetto et al. (2005) with the blood glucose meter (Accu-Chek Active, Roche Diagnostics Laboratory 2002). For the 2nd method, blood (0.5 ml) was placed in heparinized tubes and glucose was determined using the enzymatic method (glucose oxidase/peroxidase) by spectrophotometry. Three experiments were conducted:

1st experimentation

Glucose kinetics was performed on 20 rats of 5 groups; glucose was taken at t0 (basal glucose), t60 min, t90 min, t120 min and t180 min.

2nd experimentation

A blood sample taken from the saphenous vein after deep anesthesia (infiltration of 0.5cc of xylocaine 2%), skin incision and exposed surgically vessel was made at t0 for the 20 rats in the 5th groups. The glucose was performed with spectrophotometry.

3th experimentation

At the 16th day, the test of oral glucose tolerance was made alive in rats, fasted for a day. After gavage with physiological saline (for controls), serum glucose 50% (for other rats) and butanol extract to the batch. After half an hour, blood samples were performed by intra-cardiac-after stilling the acepromazine (vetranquil 1%) at a dose of 0.3 cc / animal (IM) and caudal puncture. The glycemia has been revealed by the glucometer (blood in the tail) and by enzymatic method of heparinized blood (sample intra-cardiac puncture). The animals were then sacrificed by intracardiac injection of 0.75 cc of xylocaine (lidocaine 1%).

Table 1. Effets of *Erythreae centaurium* extracts on glycemic index in normal and hyperglycemic treated rats at different time intervals.

Extract	Time (min)				
	t_0	t_{60}	t_{90}	t_{120}	t_{180}
Control	82.5±4.51	96.25±12.87	99.00±8.83	81.50±19.64	85.00±16.21
Variation (%)	100	116.67	120	103.62	103
H.G.A.E.	109.5±31.04	129.0±11.22	122.25±32.27	125.0±29.22	126.5±22.9
Variation (%)	100	118.34	112.16	114.68	116
H.G.B.E.	198.25±55.79	220.0±71.67	151.75±36.77	130.5±43.15	133.0±39.02
Variation (%)	100	110.97	76.54	65.82	65.82
H.G.D.	116.00±30.43	114.5±29.65	76.54±4.97	72.0±10.71	68.0±7.70
Variation (%)	100	98.70	72.41	62.06	58.62

H.G.A.E.: Hyperglycemic + aqueous extract; H.G.B.E.: hyperglycemic + butanolic extract; H.G.D.: hyperglycemic + daonil®.

Table 2. Effets of *Erythreae centaurium* extracts on glycemic index in hyperglycemic treated rats and control group at different time intervals.

Extract	Time (min)				
	t_0	t_{60}	t_{90}	t_{120}	t_{180}
Hyperglycemic	82.25±9.39	104.75±25.37	93.25±11.35	88.00±9.09	84.25±10.94
Variation (%)	100	122.87	109.38	103.22	98.82
H.G.A.E.	109.5±31.04	129.0±11.22	122.25±32.27	125.0±29.22	126.5±22.9
Variation (%)	100	117.80	111.64	114.15	115.52
H.G.B.E.	198.2±55.79	220.0±71.67	151.75±36.77	130.5±43.15	133.0±39.02
Variation (%)	100	110.97	76.54	65.82	65.82
H.G.D.	116±30.43	114.5±29.65	76.54±4.97	72.0±10.71	68.0±7.70
Variation (%)	100	98.70	72.41	62.06	58.62

H.G.A.E.: Hyperglycemic + aqueous extract; H.G.B.E.: hyperglycemic + butanolic extract; H.G.D.: hyperglycemic + daonil®.

Statistical analysis

The results are expressed as mean values along with their standard error of the mean (SEM). The overall statistical analysis of our results, the test of analysis of variance (ANOVA) followed by DUNNET test was used to determine statistical significance. *P* is accepted for a value equal to or less than 0.05.

Histological study

The samples were performed on different organs (liver, kidneys) for dead animals (11) and sacrificed animals (9). They were fixed in 10% formalin (37% formaldehyde) for a week and then histological investigations were performed.

RESULTS AND DISCUSSION

Glycemic evaluation

The results of the variation in glucose levels are shown in Tables 1, 2, Figures 1 and 2. The administration of *Erythrea centaurium* L. (pers.) at 0.66 ml/100 g body weight (aqueous extract), and 0.015 ml/100 g (butanolic extract) body weight and 0.25 mg/100 g body weight of Daonil® has reduced significantly glycemia compared to controls at t60, t90, t120 and t180 min. Glycemia obtained with Daonil® was significantly lower than that of (HG + BE) and (HG + AE) groups from t60 until t180 min. The difference between the (HG + AE) and (HG + BE) was most important at t0, t60 and t90 min. Data from this study showed that aqueous extract (0.66 ml/100 g body weight) and especially the butanolic extract (0.015 ml/100 g body weight) of *E*. *centaurium* L. (pers.) reduced blood glucose levels in normoglycemic and hyperglycemic rats. According to Alaoui et al. (1992), an association of three plants (*Ammi visnaga*, *Thymus ciliates* and *E. centaurium*) used in traditional medicine in Morocco has demonstrated its hypoglycemic effect in normoglycemic rats. The same plants, dried and powdered are combined in the following proportions (*A*. *visnaga* 20 g, *E*. *centaurium* 20 g and 5 g of *T*. *ciliatus*) are administered orally in the form of aqueous extract at a dose of 450 mg/kg to rats subjected to HPVO. This aqueous extract showed some anti hyperglycaemic activity against hyperglycemia induced by glucose in 30 min after administration and short-term (4 h), the aqueous decoction is significantly active. The aqueous decoction contains the association thus contains water-soluble ingredients (Alaoui et al., 1995).

Our results are also similar to those of other authors who observed that administration by gavage of 1 ml/100 g

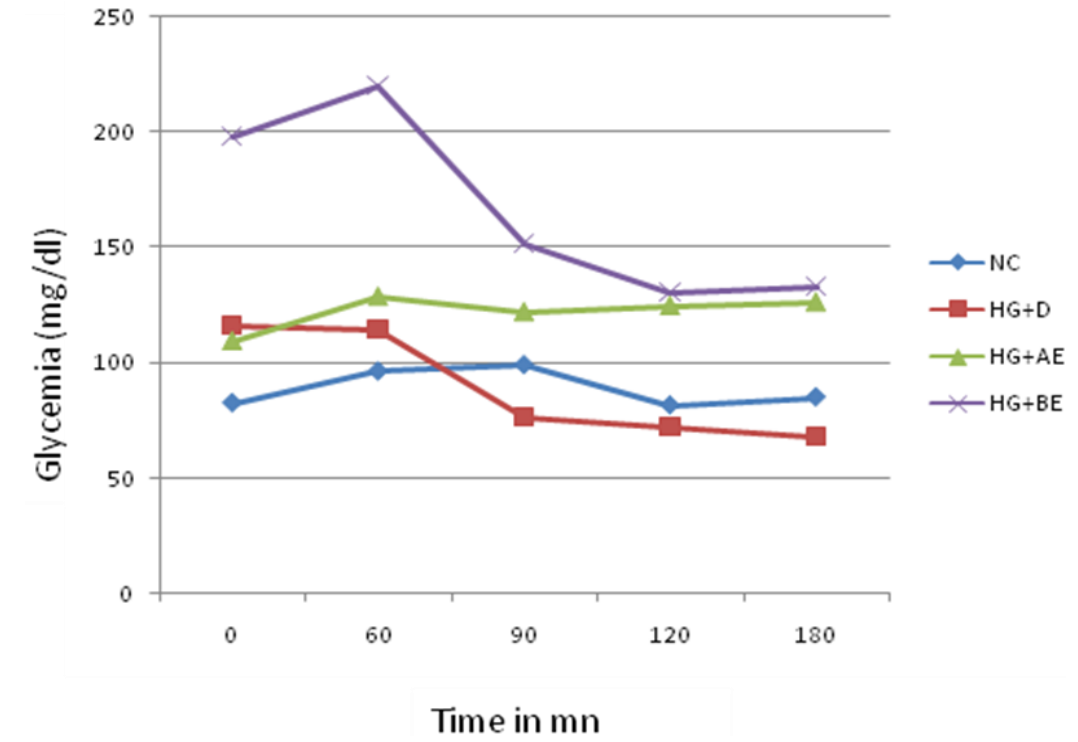

Figure 1. Glycemia of the different groups compared to normal control rats. NC: Normal control; HG + AE: hyperglycemic + aqueous extract; HG + BE: hyperglycemic + butanolic extract; HG + D: hyperglycemic + daonil®.

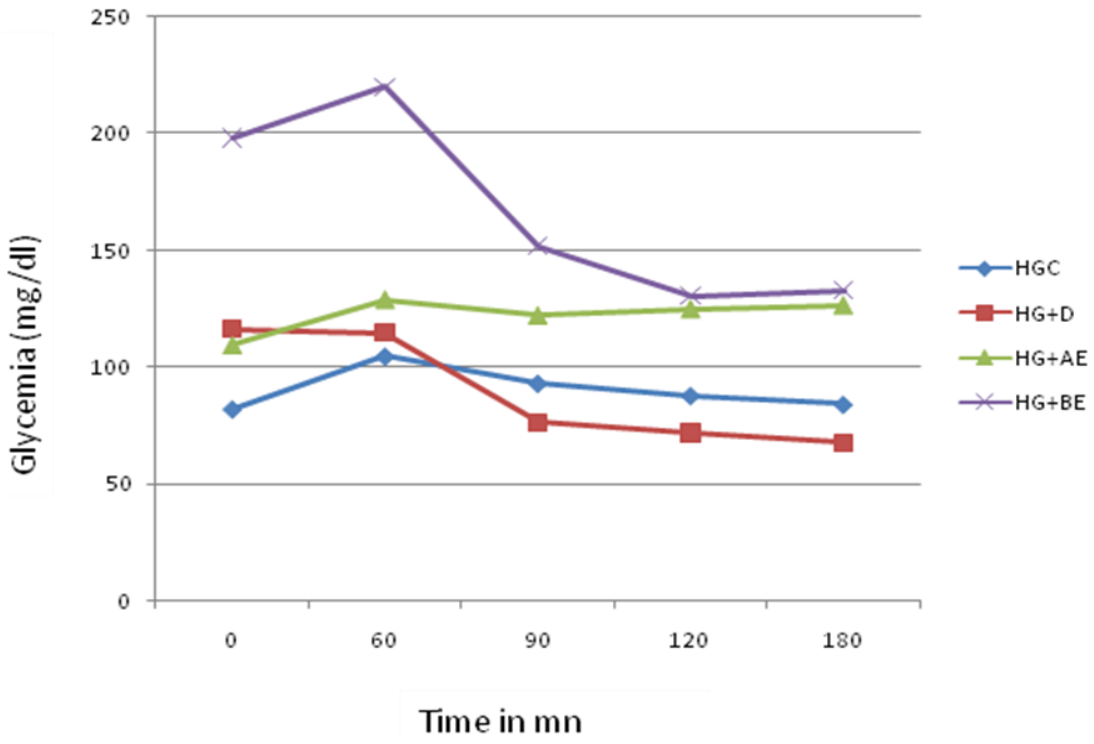

Figure 2. Glycemia of the different groups compared to the hyperglycemic control rats. HGC: Hyperglycemic control; HG + AE: hyperglycemic + aqueous extract; HG + BE: hyperglycemic + butanolic extract; HG + D: hyperglycemic + daonil®.

body weight of an aqueous decoction of 20% of each of the following plants: *Marrubium vulgare* L., *Artemisia herba-alba*, *Olea europaea* and *Zygophyllum cornutum* rats subjected to an overload of glucose HPVO revealed interesting hypoglycemic properties. The hypoglycemic effect of the extract of *Z*. *cornutum* is the most important, with a decrease in blood glucose by 46% at 60 min followed by *M*. *vulgare* L. (Hmamouchi et al., 1995). A study has evaluated hypoglycemic activity of *Zygophyllum Globularia alypum* and *gaetulum*; oral administration of these plants at a dose of 0.7 g/kg produced hypoglycemia in normoglycemic and hyperglycemic rats (Skim et al., 1999). The leaves of *Cogniauxia podoleana* also were tested for hypoglycemic activity in rats, a reduction of 21% was observed 3 h after administration by gavage to rats subjected to HPVO extracts unheated and heated administred at 250 and 500 mg/kg body weight respectively (Diatewa et al., 2000). *Anacardium occidentale* L. is a plant traditionally used in southern Cameroon to treat diabetes; the administration of the aqueous extract of the leaves of this plant, at a dose of 175 and 250 mg/kg body weight in rats after glucose tolerance test was driven attenuation of blood glucose after 1 h (Kamtchouing et al., 1998; Sokeng et al., 2001).

In a study of Sefi et al. (2011), the authors have tested the leaf extract of this plant in diabetic rats (200 mg/kg bw/day, i.p for 30 days); their study has concluded that *Centaurium erythrea* treatment exerts a therapeutic protective effect in diabetes by decreasing oxidative stress and pancreatic β-cells' damage which may be attributed to its antioxidative potential. Another study of Hamza et al. (2010) conducted on diabetic male mouse induced with a standardised high fat (HFD) diet, has shown a preventive effect of *C. erythrea* extract on HFD-induced diabetes at a dose of 2 g/kg body weight daily for 20 weeks. The results of our study conducted on rats confirm the anti hyperglycemic activity of small centaury in mouse showed in the study of Hamza et al. (2010). We mention that the material plant in these two studies is from the same region (East of Algeria).

Lesions

Before the last experiment, 11 animals died within 15 days following the 1st and 2nd experiment. These animals were autopsied and histological samples of liver and kidney were made. After the 3rd experiment, all animals were killed and necropsied. The lesions found in the majority of dead animals, are congestion and hemorrhage organs (liver, kidney) and the corpse. Necrotic lesions of the liver and cystic kidneys were observed. Microscopic lesions are essentially lesions degeneration and necrosis of the liver and especially kidney degeneration (epithelium with occasional involvement of the glomerulus). Lesions congestion and hemorrhage are the signs of intoxication. It seems that the dose recommended by traditional healers is too high, at least in rat's model, which has caused the death of animals and the observation of these lesions or toxicity reported elsewhere; but for some authors, toxicity does not exist for this plant (Mroueh et al., 2004). Kidney damage due to the administration of centaury has already been reported by other authors (Haloui et al., 2000). It seems that the dose given to man by traditional healers is too high, at least in rats. We mention here a study of Tahraoui et al. (2010) conducted in rats and mice to determine potential toxicity of this plant. In acute toxicity, the authors have tested by gavage the doses of 1 to 15 g/kg (single dose in mice) and in sub-chronic studies, they have tested orally in rats 3 doses 100, 600 and 1200 mg/kg daily for 90 days. The searchers have concluded that the *E*. *centaurium* -extract is relatively non-toxic in view of their obtained results.

Conclusion

The experimental study in rats confirmed the anti-hyperglycemic effect of small centaury (*E*. *centaurium*) at a dose of 0.66 ml/100 g body weight of the aqueous extract and 0.015 ml/100 g body weight of butanolic extract and noted that the plant has side effects for a long time, at least at the tested dose. Lesions are generalized congestion, degeneration and necrosis of the liver and especially kidney. This dose, given to man by traditional healers, is too high, at least in rats. The toxicity of high-dose of this plant has already been reported by some authors.

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Full Length Research Paper

Association of plasma protein C levels and coronary artery disease in men

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Several studies have shown the risk factor causes of coronary heart disease. In this study we tested the hypothesis that plasma protein C level might be used as a biomarker for coronary heart disease and myocardial infarction. The study included 60 men that were classified into 3 groups according to clinical examination; group I set as healthy control group, group II set as patients with ischemic heart disease and group III set as patients suffering from myocardial infarction. Different parameters were measured including, coagulation factor prothrombin time, partial thromboplastin time, fibrinogen and protein C. The activity of the cardiac enzymes (creatine phosphokinase, creatine phosphokinase-MB and lactate dehydrogenase) was also measured. Finally, lipids profile (total lipids, phospholipids, triacylglycerol, total cholesterol, low density lipoprotein cholesterone (LDL-C) and high density lipoprotein (HDL-C) were measured. The results demonstrate significant decrease level of protein C and prothrombin concentration (%) in ischemic heart disease and in myocardial infarction (MI) groups, when compared to the control group. Meanwhile, MI group showed more significant decrease comparing to IHD. Plasma protein C might serve as a marker for coronary artery disease in men. Further studies are warranted to bolster the data and to identify pathogenesis links between innate immune system activation and atherosclerosis.

Key words: Ischemic heart disease, myocardial infarction, protein C, coagulation factor, lipids profile.

INTRODUCTION

Atherosclerosis is a condition in which there is an artery wall thickness as a result of the accumulation of fatty materials such as cholesterol. It is a syndrome affecting arterial blood vessels, a chronic inflammatory response in the walls of arteries, caused largely by the accumulation of macrophage white blood cells and promoted by low density lipoprotein (plasma proteins that carry cholesterol and triglycerides) without adequate removal of fats and cholesterol from the macrophages by functional high density lipoprotein (HDL). It is commonly referred to as a hardening of the arteries. It is caused by the formation of multiple plaques within the arteries (Finn et al., 2010).

Myocardial infarction (MI) commonly known as a heart attack, results from the interruption of blood supply to a part of the heart, causing heart cells to die. This is most commonly due to occlusion of a coronary artery following the rupture of a vulnerable atherosclerotic plaque in the wall of an artery (Didangelos et al., 2009). Moreover, may be a minor event in a lifelong chronic disease, it may even go undetected, but it may also be a major

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Abbreviations: HDL, High density lipoprotein; **MI,** myocardial infarction; **IHD,** ischemic heart disease; **O-LDL,** oxidized low density lipoprotein; **ECG,** electrocardiogram; **CPK,** creatine phosphokinase; **CPK-MB,** creatine phosphokinase MB; **APC,** activated protein C; **CAD,** coronary artery disease; **CVD,** cardiovascular disease.

catastrophic event leading to sudden death or severe hemodynamic deterioration. Myocardial infarction may be the first manifestation of coronary artery disease, or it may occur, repeatedly, in patients with established disease (Thygesen et al., 2007). Disturbed lipid profile is one of the most important and potent risk factors in ischemic heart disease (IHD). It has been demonstrated that raised oxidative stress promotes several undesirable pathways including the formation of oxidized low density lipoprotein (O-LDL) and oxidized cholesterol which encourages cholesterol accumulation in arterial tissues (Maharjan et al., 2008)

Excessive consumption of saturated fat and cholesterol has been linked with increased concentration of plasma fibrinogen, a major risk factor for thrombosis that leads to heart attacks and strokes (Avogaro et al., 1988). Abnormalities in the blood coagulation factors regulating thrombosis may also contribute to the risk and extent of thrombosis (Hamker et al., 1991).

Homeostasis is a complex physiologic process involving a promoting factor (procagulants) counter-balance by naturally occurring inhibitors. Derangement of this balance is considered to play an important role in the pathogenesis of thrombosis (Kenneth, 1992). Recently, it was found that heparin coagulation factor (HCII) inhibits thrombin activity by binding to derma tan sulfate and has been shown to be a novel and independent risk factor for atherosclerosis (Huang et al., 2008).

Human protein C is a vitamin K-dependent glycoprotein structurally similar to other vitamin K-dependent proteins affecting blood clotting, such as prothrombin, factor VII, factor IX and factor X. Protein C, also known as autoprothrombin IIA and blood coagulation factor XIV, is a zymogenic (inactive) protein, the activated form of which plays an important role in regulating blood clotting, inflammation, cell death and maintaining the permeability of blood vessel walls in humans and other animals (Mosnier et al., 2007).

In addition, activated protein C accelerates fibrinolytic activity by raising the level of plasminogen activator or by decreasing the level of plasminogen activator inhibitor (Zateishchikov et al., 1990). The determination of protein C makes it possible to identify patients who are at risk of thrombosis so that preventive measure can be instituted (Sturk et al., 1987). We tested the hypothesis that plasma protein C and different coagulation factors might be able to be used as a biomarker for coronary artery disease (CAD).

MATERIALS AND METHODS

Subjects

Sixty male subjects were selected from Health Insurance Hospital, Al-Azhar University, Cairo Egypt. Their ages ranged between 40-65 years old. They were classified into three groups after complete history taking and through clinical examinations and full investigations by electrocardiogram (ECG), echo, chest X-ray, and laboratory ratory examinations. Group I: Control group, twenty normal healthy men with no history for disease and drug intake. Group II: Twenty cases were suffering from ischemic heart diseases (IHD). Group III: Twenty cases were diagnosed as having MI disease. None of the study participants had any of the following disorders, associated with an acute phase reaction, febrile acute infection or acute state of a chronic infection or an inflammatory disease, underlying hematologic or malignant diseases and renal disorders. Current medication and sociodemographic characteristics were also recorded. Participation was voluntary, written informed consent was obtained from each subject upon entry into the study. The study was approved by the ethics committee of the University of Al-Azher.

Blood sampling

Ten ml of blood were collected from each subjects, 5 ml blood were added to 3.8% trisodium citrate solution, in the proportion of 9 volumes of blood to one volume of anticoagulant solution and centerifugated at 3000 rpm for 10 min. The plasma was separated for determination of the following parameters: Activated prothrombin time (PT.second) according to Loeliyer et al. (1985). Activated partial thromboplastin time (PTT.second) was measured according to Munteam et al. (1992). Also plasma fibrinogen g/L and activated protein C% were measured according to Exner and Voasoki (1983).

The other 5 ml of blood were let to clot and the serum was used to determine the following parameters, activity of the enzyme creatine phosphokinase (CPK) according to the study of Szasz (1976) and the activity of the isoenzyme creatine phosphokinase MB (CPK-MB) (Szasz, 1976).

Also the activity of the enzyme lactate dehydrogenase (LDH) (Anon, 1977) was measured. Serum total lipid, phospholipids, triacylglycerol, cholesterol and HDL-C were also measured according to Knight et al. (1972), Henry (1974), Stavropoulos (1974) and Abell et al. (1952). Low density lipoprotein cholesterone (LDL-C) was measured according to the equation:

LDL-C = total cholesterol - HDL-C – TG/5

Statistical analysis

All results were expressed as mean ± S.E of the mean. Statistical Package for the Social Sciences (SPSS) program, version 11.0 (Chicago, IL, USA) was used to compare significance among three groups. Difference was considered significant when $p \leq 0.05$.

RESULTS

It is clear from Table 1 that there was a highly significant increase in the level of prothrombin time (in seconds) in group II and III when compared to the control group. Also, there was a highly significant decrease in prothrombin concentration percentage in IHD and MI groups compared to the control group.

Moreover, MI group showed a significant decrease compared to IHD group. It could be concluded from this Table that partial thromboplastin time level showed highly significant increase in MI group as compared to IHD group. Plasma fibrinogen increased significantly in IHD and MI groups compared to the control group. Furthermore, IHD group showed lower value of plasma fibrinogen as compared to MI group. Finally, there was a highly

Table 1. Clinical Characteristics and Coagulation Factors in Control, IHD and MI groups.

Parameter	Control group (n=20)	IHD (n=20)	MI (n=20)
Age (yrs)	50.0 ± 11	60 ± 9.5*	56.0 ±10.0
BMI (Kg/m^2)	27.0 ± 3.5	27.8± 3.4	27± 3.5
Current smoker, n (%)	6 (30.0)	15(75.0)	13 (65.0)
Prothrombin time (seconds)	12.4 ± 0.33	14.94± 0.84*	16.17 ± 1.09*§
Prothrombin conc.%	91.88 ± 6.13	57.6 ± 8.41*	49.7 ± 7.3*§
Partial thromboplastin (seconds)	33.74± 2.97	38.64 ± 6.99*	50.47± 6.24*§
Plasma fibrinogen (mg %)	262.8 ± 14.04	306.55 ± 7.33*	346.45 ± 16.63*§
Activity of Protein C (%)	109.53 ± 5.07	81.9 ± 7.77*	62.35 ± 5.13*§

Data are presented as mean ± SD, *p <0.05 vs. control, § p <0.05 significant (IHD) vs. (MI).

Table 2. The activity of cardiac enzymes in control, IHD and MI groups.

Parameter	Control group (n=20)	IHD (n=20)	MI (n=20)
Creatine phosphokinase (U/L)	90.15 ± 19.98	201.8 ± 13.82*	679.9 ± 17.807*§
Creatine phosphokinase –MB (U/L)	10.72 ± 2.62	35.45 ± 6*	107.25 ± 5.487*§
Lactate dehydrogenase (U/L)	114.1 ± 2.81	133.05 ± 3.047*	361.6 ± 10.473*§

Data are presented as mean ± SD, *p <0.05 vs. control, § p <0.05 significant (IHD) vs. (MI).

significant decrease in the levels of protein C in IHD and MI group as compared to control group. Meanwhile, MI group showed significant decrease in protein C level compared to IHD.

It could be seen from Table 2 that there was a highly significant increase in level of CPK, CPK-MB, and lactate dehydrogenase concentration in MI group as compared to IHD group. Also both IHD, and MI groups showed a highly significant increase compared to the control group. The results illustrated in Table 3 showed that lipid profiles (total lipids, total cholesterol, LDL-C) in MI and IHD groups were highly significant increased as compared to the control group, while HDL-C decreased significantly in these groups.

MI groups showed a significant increase in lipids profile as compared to IHD. The present study showed no significant change in phospholipids concen-tration in IHD group while there was highly significant increase in MI group as compared to the control group.

DISCUSSION

Prothrombin time (PT) measured the clotting time of plasma in the presence of an optimal concentration of tissue extract (thromboplastin) and indicates the overall efficiency of the extrinsic clotting system. The test depends on reactions with factors V, VII, and X and on the fibrinogen concentration of plasma (Dacie and Lewisi, 1991). The results of this study are in agreement with that of Gupta et al. (1997) and Folsom et al. (1997). Also, our results are in accordance with the study of Erbay et al (2004). The elevation of fibrinogen and prothrombin levels acts as a risk factor and may play a causative role in cardiovascular disease. Also, Chambliss et al. (1992) reported highly activated partial thromboplastin time and plasma factor VIII but decrease in value of protein C and antithrombin III activity. Also Kenneth et al. (1992) indicated that plasma fibrinogen concentration factor VII, protein C and antithrmboplastin III levels were signify-cantly higher in early atherosclerosis in carotid arteries which may be a useful marker for identifying individuals at high risk of developing arterial disorders.

In addition, this work showed a highly significant decrease in plasma protein C levels in IHD and MI group as compared to the control group. These results are in agreement with that obtained by Lauribe et al. (1992). The raised fibrinogen and decreased protein C appeared to be risk factor for sudden cardiac death. Gibbs et al. (1992) reported increase in the procoagulants fibrinogen, factor VIII, and decrease in protein C and antithrombin III in cases of myocardial infarction. Treatment with activated protein C significantly improved hemodynamic after ischemia-reperfusion and reduced ischemia-reperfusion-induced myocardial apoptosis in rats (Pirat et al., 2007). Henkens et al. (1993) observed that thromboembolic events occurred in 30% of protein C deficient and in 35% of protein S deficient persons. Also, Dahl back et al. (1993) reported poor anticoagulant response to activated protein C in several families with hereditary tendency to venus thrombosis. Moreover, It was also reported that concentration of protein C level

Table 3. Lipids profile levels in control, IHD and MI groups (Mean ± SD).

Parameter	Control group (n=20)	IHD (n=20)	MI (n=20)
Total lipids (mg %)	628.8 ± 42.81	850.25± 50.43*	982.62 ± 84.38*
Phospholipids (mg %)	207.8 ± 22.84	231.25± 12.84	250.8± 6.44*§
Triacylglycerol (mg %)	125.9 ± 20.67	174.3± 6.891*	185.5 ± 13.4*§
Total cholesterol (mg %)	191.95 ± 15.22	303.6 ± 20.35*	329.35 ± 22.88*
HDL-C (mg %)	55.2 ± 6.12	42.4 ± 4.68*	41 ± 4.21*
LDL-C (mg %)	107.35 ± 7.35	222.5 ± 16.2*	247.75± 19.32*

Data are presented as mean ± SD, *p <0.05 vs. control, § p <0.05 significant (IHD) vs. (MI).

and activity of protein C deficiency has a bearing with pulmonary infarction.

Van-der-Ban et al. (1996) found a reduced response to activated protein C (APC) is associated with an increased risk for cerebrovascular disease but not with an increased risk for myocardial infarction. Goto et al. (1992) reported augmented plasma protein C activity after coronary thrombolysis with urokinase in patients with acute myocardial infarction, thus, it was suggested that urokinase administration for coronary thrombolysis not only causes fibrinolysis, but also induces thrombin activity which may be antagonized by augmented intrinsic protein C activity. The diagnosis of MI is established in patients with chest pain and equivocal electrocardiogram changes by demonstrating a rise in blood levels of creatine kinase MB (CK-MB) and/or an increase in cardiac troponin I (cTnI) or cardiac troponin T (cTnT). Previous studies have shown that levels of CK-MB are increased in the left ventricle of individuals with heart disease Welsh et al, (2002).

While CK-MB as a cardiac marker depended on its relatively high concentration in heart muscle (>20%) compared to typical skeletal muscle (1–2%). There is evidence that higher concentrations of CK-MB in heart may result from ischemic stress. For example, concentrations of CK-MB have been found to be significantly higher in heart muscle of experimental animals and human myocardium with coronary artery disease, aortic stenosis, or heart failure, compared to normals. A number of studies have shown that the concentration of CK-MB is higher in ventricular myocardial tissue in animal models of hypertrophy or ischemia and in humans with a variety of cardiac conditions, compared to controls or young individuals without cardiac disease. In human myocardial biopsy material, concentrations of CK-MB have been reported to be 100-fold greater in hearts from patients with aortic stenosis, coronary artery disease, and coronary artery disease with left ventricular hypertrophy compared to patients without such findings (Welsh et al., 2002).

The study of many enzymes activities are valuable in diagnosis of many disease as the rise in the serum enzyme of CPK and CPK-MB and lactate dehydrogenase are commonly used for diagnosis of coronary heart disease. In the present study, it was found that there was a highly significant increase of serum creatine phosphorkinase CPK, CPK-MB and serum lactate dehydrogenase levels in IHD and MI groups as compared to the control group. The discovery of isoenzyme determination has improved the diagnostic value of enzyme tests. The cardio specific isoenzyme of CK (CK-MB) has been used successfully for the detection of myocardial infarction. Our results are in agreement with that of Welsh (2002) and Kato et al. (2006). The European Society of Cardiology (ESC) and American College of Cardiology (ACC) state that any elevation, however small, of a troponin or the creatine kinase MB (muscle, brain) isoenzyme is evidence of myocardial necrosis and that the patient should be classified as having myocardial infarction, however small (Antman et al., 2004).

Hyperlipidemia refers to increased levels of lipids (fats) in the blood, including cholesterol and triglycerides. Although hyperlipidemia does not cause you to feel bad, it can significantly increase your risk of developing coronary heart disease, also called coronary artery disease or coronary disease. People with coronary disease develop thickened or hardened arteries in the heart muscle. This can cause chest pain, a heart attack, or both (Saunders, 2007). Hyperlipidemia is a disturbance of the lipid transport system that results from abnormalities in the synthesis or degradation of plasma lipoprotein (Brown and Goldstein, 1983). There is strong evidence between abnormalities of lipids metabolism and gradual change of atherosclerosis and coronary heart disease. The substance that gives the atheroma its character is the lipid, chiefly cholesterol esters (Roheim, 1986). Measurements of plasma lipid and lipoprotein levels have been used in diagnostic medicine to assess the risk of coronary artery disease. Cholesterol and triglycerides levels have been recognized as predictors of CAD. HDL-C and LDL-C have been considered the most accurate indicators of CAD. Increased level of LDL cholesterol is associated with increased incidence of CAD.

Our study reveals a high significant increase in serum total lipids in IHD and MI compared to control group. This is in accordance with the results of Brown and Goldstein (1983). They noted the increase in serum total lipid in CAD patients. Also, it was reported that hypertension, smoking and hyperlipidemia are the most important risk factors of IHD. Saturated fat intake has been linked to an increased risk of cardiovascular disease (CVD), and this

effect is thought to be mediated primarily by increased concentrations of LDL cholesterol (Patty et al., 2010). The present study shows no significant change in phospholipids concentration in IHD group while there was highly significant increase in MI group as compared to the control group. Natio (1988) proved that the ratio of phospholipids to cholesterol ester level resulted in a corresponding change in phospholipids in similar direction.

Furthermore, triacylglycerol increased significantly in both IHD and MI as compared to the control group. It was suggested by Despres et al. (1990) that triglyceride molecules are not themselves atherogenic. High plasma triacylglycerol level may indirectly represent a cardiovascular risk factor through its effect on lipoprotein composition. Also, the results are in agreement with those of Jensen et al. (1991) who found that triglyceride level were higher in CAD patients and severity of coronary atherosclerosis has been shown to correlate better with serum concentration of triglyceride than of cholesterol.

Sigurdsson et al. (1992) suggested that elevated triglyceride levels are important as a risk factor only when associated with other lipoprotein abnormalities (elevated LDL-C or decreased HDL-C). Also Welin et al. (1991) demonstrated from follow up of incidence of coronary heart disease increased 5 fold from the lowest to the highest of value triacylglycerol. Increased serum triglycerides are a major coronary risk factor in elderly men. Moreover, Assmann (1992) suggested that triglyceridemia is a powerful additional coronary risk factor when excessive triacylglycerol coincide with a high ratio of plasma LDL-C to HDL-C.

In this study, there was a highly significant increase in serum total cholesterol in IHD and MI as compared to control group. The present study is in agreement with Kondreddy et al. (2010) who found that total cholesterol (TC) and LDL-C were significantly increased while HDL-C was significantly decreased among the CHD group. This is in accordance with the results of Bainton et al. (1992). They found that total cholesterol was higher in CAD patients than normal controls. Also, Swedarsen et al. (1991) showed that hypercholesterolemia without associated hypertriglyceridemia was the commonest abnormality. Jensen et al. (1991) observed that plasma cholesterol above the level approximately 270 mg% proportionally increased of CAD. It was concluded by Bainton (1992) that cholesterol was a more important risk factor than HDL-C and was considered to be the most important single lipid risk factor in men. The level of the plasma lipoprotein play an important role in the pathogenesis of atherosclerosis, particularly low level of HDL-C and high level of LDL-C (Roheim, 1986).

In this work the HDL-C levels showed highly significant decrease in both IHD and MI groups as compared to control group. A similar result was found by Duval et al. (1989) who reported that there was s significant decrease in HDL in cardio vascular disease. Pometta et al. (1987) reported that HDL-C concentration correlated inversely to the development of atherosclerosis; therefore they were considered to be negative risk factor. Finally, there was a highly significant increase of LDL-C in IHD and MI groups comparing to the control group. Similar results were obtained from Henriksen (1984) who found that the lipids in atherosclerotic lesion are derived from plasma LDL. Also Jensen et al. (1991) found that LDL was higher in CAD patients than control. Avogaro et al. (1988) and Badimon et al (1992) studied LDL metabolism and proved that LDL seems to be responsible for transported approximately 70% of the total cholesterol by LDL-C the liver to the tissues.

In conclusion, this study demonstrates the association between protein C and another coagulation factor and other important risk factors as lipid profile and plasma protein C might serves as a marker for coronary artery disease in men.

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Full Length Research Paper

Emerging *Acinetobacter schindleri* in red eye infection of *Pangasius sutchi*

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This communication provides an insight into the emerging of new infection "red eye" in *Pangasius sutchi* and aimed to screen the prime pathogens involved in disease. The pathogen was isolated from diseased *P. sutchi* and characterized by morphological, biochemical and molecular approach, which includes 16s r RNA gene sequencing. Polymerase chain reaction (PCR) amplified 16s RNA was separated using agarose gel electrophoresis, eluted product was sequenced and BLAST analysis was carried out to identify the pathogens. Identified virulent bacterial strain *Acinetobacter schindleri* with LD_{50} $10^{8.35}$ initiated re-infection in experimentally in infected *Pangasius* fingerlings. This study provided the evidence of *A. schindleri* which is true causative agents in red eye disease in *P. sutchi*. To the best of knowledge of this study, there was no track record of *A. schindleri* eye infection in fishes till date around the globe.

Key words: *Pangasius sutchi*, 16s r- RNA gene sequencing, *Acinetobacter schindleri,* LD_{50}.

INTRODUCTION

Pangasius sutchi is the exotic fish introduced in India from Thailand because of its high commercial value. The farmers of Janardhanapuram, Nandivada (Md), Krishna (Dist), Andhra Pradesh, culturing *Pangasius* in fresh water as intensive, monoculture with stock density of 50,000 per hectare, fed with floating feed having 20 to 23% protein, feeding rate up to 1.2 to 1.6% 10^3 kg body mass of fishes. *P. sutchi* is highly resistant species, and it is voracious feeder shown good food conversion rates (FCR) and give maximum sustainable yields (MSY) in short period. Nutrient rich feeds leave higher concentrations of ammonia and nitrite in culture waters and these stresses the fish in enormous rate and make them susceptible to different diseases. Culture waters with high organic matter pollute not only the tank, but also surroundings, and support the growth of many pathogens. For *Pangasius,* the most important bacterial diseases are bacillary necrosis; red spot have been reported by (Tu et al., 2008). These pollution problems may support the growth of *Acinetobacter* members in culture waters.

Acinetobacter members are found in water and act as common flora (José Américo, 2001; Marian, 1990). The alimentary tract of fresh water trout has *Acinetobacter* members (Trust, 1974). A significant increase in the microbial load of *Acinetobacter* members in ponds treated with different chemotherapeutics has been reported by Andreas Petersen (2002) and their entrance into culture waters

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Abbreviations: FCR, Food conversion rates; **MSY,** maximum sustainable yields; **PCR,** polymerase chain reaction; **SDS,** sodium dodecyl sulfate; **CTAB,** cetyl trimethyl ammonium bromide; **BOD,** biological oxygen demand; **COD,** chemical oxygen demand.

along with contaminated feed has been reported by Trevors et al. (1977). Although *Acinetobacter spp* acts as a severe human pathogen, there are only few studies to date that report it as a pathogen for fish. The genus *Acinetobacter* show wide range of distribution, recovered from soil, water, living organisms. The bacteria very quickly became important member in bacteria landscape in hospitals, responsible for number of nosocomial infections in humans like surgical wound, urinary tract, respiretory tract (Wolff et al*.,* 1997), pneumonia, secondary meningitis (Bukhary et al*.,* 2005), endocarditis (Levi and Rubinstein, 1996), peritonitis, skin and soft tissue infections (Fierobe et al., 2001). Kalidas Rit and Rajdeep (2012) have reported *Acinetobacter* sp and their member's cause nosocomial infections and susceptibility patterns for different antibiotics. The *Acinetobacter* members show resistance to wide range of antibiotics like ampicillin, carbapenems (Mussi et al*.,* 2005), carbenicillin, cephalosporin's (Heritier et al., 2006), amino glycosides, fluoroquinolones (Vila et al., 1993), carboxy pencillins (Joly and Guillov et al., 1995). They produce a wide range of amino glycoside inactivating enzymes (Buisson et al., 1990). Nemec (2001) reported *Acinetobacter schindleri* infections in human nosocomial infections, and Bouvet and Grimont (1986) firstly reported *A. haemolyticus* infections in humans. Emerging of new multi drug resistant bacterial pathogen, *Acinetobacter baumannii* associated with snake head *Channa striatus* eye infection has been reported by Rauta et al. (2011). The present study aimed to identify pathogens at molecular level from diseased *P. sutchi* suffering from red eye infection and to be proved as primary agents in disease.

MATERIALS AND METHODS

Collection of water and diseased fish samples

Diseased moribund fish samples (10) were collected from above said locality ponds, and brought to the laboratory. The fishes show different symptoms like gill impairment, erythro dermatitis, petechiae at lateral line, red mouth, redness at fin bases, swollen red colour anus, pop eye, red arched region around eye, swollen enlarged liver in light yellow colour, shrunken gastro intestinal tract and spleen, and hemorrhages on internal body cavity. Three water samples were collected from sequential days of 15 for 45 days to be checked the parameters like water temperature, pH, ammonia, nitrite, calcium, magnesium, alkalinity, hardness, chlorides, total dissolved solids, conductivity, and dissolved oxygen (APHA, 1988).

Isolation and identification of bacteria

A loop full of sample was collected with the help of inoculation loop from eye transferred on to Rimler Shots agar medium (Hi media, Mumbai). The plates were incubated at 37°C for 24 h. The nature of the cell wall of isolate was tested by gram staining method. For further differentiation, the culture was tested for biochemical characteristics with the Enterobacteriaceae kit (Hi media, Mumbai) as per manufacturer instructions. Later organisms were subjected for molecular characterization, to differentiate organisms up to species level.

DNA extraction

Extraction of genomic DNA and polymerase chain reaction (PCR) mediated amplification of the 16s r RNA gene of bacterial strain was carried out as per the method described by Neal Stewart et al*.* (1993). DNA from saturated bacteria liquid cultures was extracted by above said methodology, includes collection of bacterial cell pellet by centrifugation, lysis of cell pellet were attained by suspending in TE buffer with 100 µg of proteinase K and 0.5% sodium dodecyl sulfate (SDS) final concentrations. After 1 h of incubation at 37°C the lysate was treated with 80 µl of 5 M NaCl and 100 µl of 10% cetyl trimethyl ammonium bromide (CTAB) solution. Cell lysate was incubated at 60°C for 10 min. Degraded proteins from the cell lysate were removed by precipitation with phenol, phenol\ chloroform and chloroform treatment, respectively. Followed by protein precipitation, bacterial genomic DNA was recovered from the resulting supernatant by iso-propanol precipitation. Precipitated DNA pellet was washed with 70% alcohol for removal of salts. The DNA pellet was allowed for air drying and re suspended in 50 µl of deionized water with 1 µl 10 mg ml¯L RNA ase A enzyme for the removal of RNA. Quality of the isolated DNA was analyzed by resolving on 1% agarose gel electrophoresis with 1X TAE buffer.

PCR amplification

The variable V3 region of DNA coding for 16s RNA was amplified by PCR with primers F- 5'- AGAGTTTGATCCTGGCTCAG –3' and R-5'- GGTTACCTTGTTACGACTT–3'. All the PCR amplifications were conducted in 50 µl volume containing 2 µl of total DNA having 54 ng per µl concentration, 200 M each of the four de oxy nucleotide tri phosphates, 1.5 µl $MgCl_2$, 5 µl of individual primers and 1 IU of Taq polymerase. The PCR amplification, used for gene amplification was consisted of initial denaturation at 95°C for 3 min, followed by 39 cycles of denaturation for 1 min at 95°C, annealing for 30 s at 56°C, and extension for 1 min at 72°C and a final extension at 72°C for 10 min. Finally, the amplified PCR product was stored at 4°C. The samples were verified on 1% agarose gel (Lonza, USA) to know Ribo print pattern. The separated bands were excised from the gel (Figure 1) by using surgical blade for elution of DNA. The elution of DNA from agarose gel was carried out as per manufacturer instructions (Real Biotech DNA/PCR purification kit CAT NO 36105).

DNA sequence and phylogenetic analysis

For sequencing analysis, amplified PCR product was sent to EUROFIN Company. All the 16s r RNA partial sequence were aligned with those of the reference micro organisms in the same region of the closet relative strains available in the Gen Bank data base by using the BLAST N facility (http:// www.ncbi.nlm.nih.gov\BLAST) and were also tested for possible chimera formation with the CHECK CHIMERA program(http://www.35.8.164.52\cgis\chimera.cgi? Su: SSU). The sequences were further analyzed by using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/). Neighbor joining phylogenetic tree (Figure 2) was constructed with the Molecular Evolutionary Genetic Analysis Package (MEGA VERSION 5.1) (Tamura K et al*.,* 2011). A boot strap analysis with 500 replicates was carried out to check the robustness of the tree. Boot strap re-sampling analysis, for the replicates was performed to estimate the confidence of the tree topologies.

Artificial challenge studies

Bacterial suspension was prepared by culturing the isolates on trypticase soy agar (TSA) plates at 30°C for 24 h and harvesting them with 50 ml of 0.85% physiological saline. Colony forming unit (CFU) per mL of this solution was determined by plating 10 fold dilu-

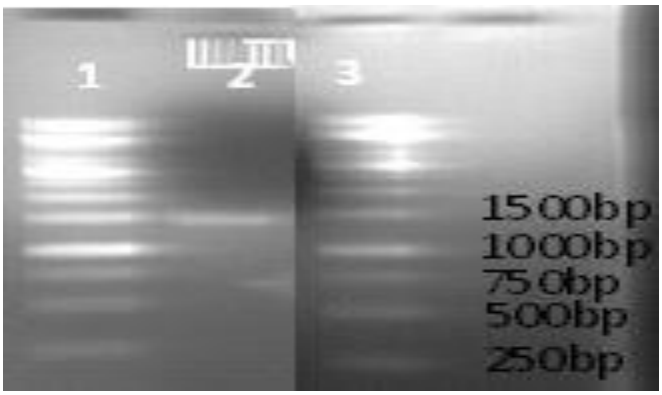

Figure 1. Ribo print pattern of isolate DNA on agarose gel.

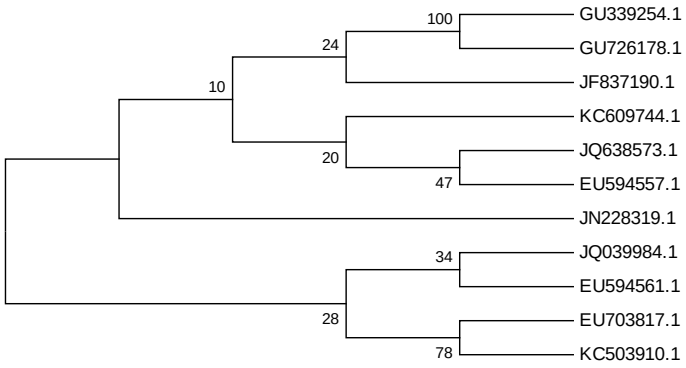

Figure 2. Neighbor-joining tree constructed using Mega 5.1 showing phylogenetic relationships of 16s RNA sequences from diseases fish to closely related sequences from Gen Bank.

Table 1. Physico- chemical parameters of water.

Parameter	Mean value± Sd
Water temperature	28.5±0.5°C
pH	8.2±0.458
Ammonia	1±0.416 mg\L
Nitrite	0.8±0.2 mg\L
Calcium	54.6±7.02 mg\L
Magnesium	91.3±6.42 mg\L
Alkalinity	473.3±30.55 mg\L
Hardness	155±5.56 mg\L
Chlorides	175.6±4.5 mg\L
TDS	1710±36 mg\L
Conductivity	1.1457±0.024 ms\cm^2
DO	1.76±0.25 mg\L

dilution series. For this purpose, the solution was diluted with distilled water. Apparently active healthy, fingerlings of *P. sutchi* (50± 10 g) were taken from the fish farm of Kaikalur, AP, India. They were stocked in 500 L cement tanks filled with fresh water and acclimatized in the laboratory condition for two weeks before starting the experiment. They were fed with standard diet in 2 divided doses daily during the experiment. Water was exchanged partially to remove left out feed and fecal matter. The lethal dose LD_{50} of the isolate was estimated according to Reed and Muench (1938). Five groups (Group1- 5) with 6 fish in each group were challenged with a series of dilutions of bacteria. The bacterial suspension prepared in phosphate buffered saline (0.15 M, pH 7.4) was injected to each fish intraperitoneally with 0.1 ml of different dilutions of bacteria. The final concentration of the bacteria injected to each was 10^5 - 10^8 CFU/mL. Control fish was injected with 0.1 mL phosphate buffered saline without bacteria. Mortality was observed till 5 days, and pathogenicity was confirmed by re-isolating the bacteria from experimentally infected fishes.

RESULTS AND DISCUSSION

The result of physico-chemical parameters of waters are presented in Table 1. Water chemistry results indicated that variation in ranges of pH, ammonia, nitrite and total dissolved solids, alkalinity, biological oxygen demand (BOD) and chemical oxygen demand (COD) show great impact on aquatic biota including fish. Inoculated fish isolates on RS medium resulted in green colour colonies are 1.5 to 2.5 mm in diameter, circular, convex, smooth and slightly opaque with entire margins. The nature of the cell wall composition of isolate was tested by gram staining method; the results confirmed the organisms as gram negative rods. The green colour colonies are positive for ONPG, lysine, ornithine, citrate, H_2S, voges proskaver, melonate, trehalose and negative for urease, phenyl alanine, nitrate reduction, methyl red, indole, esculin, melibiose, and glucose, respectively. The isolate showed good growth on nutrient agar, brain heart infusion agar, and tryptone soya agar. The isolate grew best in the temperature range of 30 to 37°C and pH 6 to 8. Biochemical results of green colonies are given in the Table 2. Sequencing analysis revealed a 100% identity with the sequence corresponding to the 16s r RNA gene of *A. schindleri YNB 103 strain* (Gen Bank accession number JQ 039984.1). Experimental infection study confirmed the pathogenicity of *A. schindleri* to *P. sutchi*. The LD_{50} of *A. schindleri* $10^{8.35}$ CFU per fish, which indicates the isolated strain, was highly virulent and capable of causing re-infection in *P. sutchi* and cause death in experimentally infected *Pangasius* fingerlings and showed similar signs even in the collected fishes from the tank outbreak (Table 3).

For effective cultivation of the fish, good quality water is needed; due to lack of sustainable management practices in water quality, fishes are prone to stress and susceptible to different diseases. All living organisms have optimum range of pH where growth is best. Water with high alkalinity not more buffered and the degree of pH fluctuation is high. Alkalinity changes can affect the primary productivity in cultured ponds. Dissolved oxygen is not at all problem to *P. sutchi* because it is air breathing fish. Elevated levels of ammonia causes gill damage and reduce the growth of fishes. Water temperature show direct impact on metabolism, feeding rates, respiratory rates of aquatic biota, and influence the solubility of oxy-

Table 2. Physical and biochemical characteristics of *A. schindleri* YNB 103.

Character	*A. schindleri*
Colony colour	Green
Gram reaction	Negative
Shape (R/C)	Rod
Motility	Non motile
Growth at different temp (°C)	
20	Negative
25	Negative
30	Positive
35	Positive
42	Positive
Growth on different media	
Nutrient agar	Positive
BHIA	Positive
Rimler-Shots agar medium	Positive
Tryptone soy agar	Positive
Growth in NaCl (w/v)	
2	Negative
4	Negative
6	Positive
8	Positive
10	Negative
Oxidative/Fermentative	Oxidative
Acid-fast test	Negative
Oxidase reaction	Negative
ONPG	Positive
Lysine	Positive
Ornithine Decarboxylase	Positive
Urease	Negative
Phenylalanine	Negative
Nitrate reduction	Negative
H_2S	Positive
Citrate	Positive
VP	Positive
MR	Negative
Indole	Negative
Production of acid from	
Melonate	Positive
Esculin	Negative
Arabinose	Variable
Xylose	Variable
Adonitol	Variable
Rhamnose	Variable
Cellobiose	Variable
Melibiose	Variable
Saccharose	Variable
Raffinose	Variable
Trehalose	Positive
Glucose	Negative
Lactose	Variable

Table 3. Lethal dose value CFU per mL of *A. schindleri*.

Group	Log dose	Death	Survived	Death	Cumulative survival	Total	Mortality ratio	Mortality (%)	LD 50
Control PBS 0.1ml	0	0	6	0	14	14	0/14	0	10^8 35 cfu/ml
CFU 10^8 2.1	0.322	2	4	2	8	10	2/10	20	
CFU10^7 3.4	0.531	3	3	3	4	7	3/7	42	
CFU$10^6$4.2	0.623	5	1	5	1	6	5/6	83	
CFU10^5 5.4	0.732	6	0	6	0	6	6/6	100	

gen. Nitrite results from feed can disrupt the oxygen transport in live fishes. Hardness of culture waters depends on levels of calcium and magnesium.

High total dissolved solids value directly indicates the presence of organic matter in culture waters. Culture water with high organic matter not only pollutes the tank, but also surrounding areas and support growth of different pathogens like causative agents of fulminant sepsis of *P. sutchi*. Some feed companies using animal meets, in place of soya while making feed pellets, it may be the indirect reason for entry of hospital landscape organisms in to aqua culture settings. Ponds treated with variety of chemotherapeutics to control different diseases, also affect the normal flora of pond bottom, it is also another reason to develop multi drug resistant bugs in to culture waters. After observing the gross symptoms of fish we postulated that emerging of new bacterial member's involvement in disease. Artificial challenge studies determined that *the* isolate can become pathogenic to *P. sutchi*. Out of 5 groups, control group fishes were injected with phosphate buffered saline, no mortality was observed, up to the end of the experiment. CFU 10^5 5.4 group shows 100% mortality of fishes within 48 h. As per Reed and Muench formula LD_{50} $10^{8.35}$ was determined for *P. sutchi*. To the best of knowledge, there was no track record of *A. schindleri* eye infection in fishes till date around the globe.

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Isolation and characterisation of *Listeria* species from ruminants in Maiduguri north–eastern Nigeria

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A cross sectional study was carried out to determine the prevalence of *Listeria* species in ruminants in Maiduguri. Three hundred faecal samples were randomly collected from ruminants at the Maiduguri central abattoir from January – March, 2011. One hundred faecal samples each were collected from cattle, sheep and goat at ante mortem by balloting comprising of fifty samples each from male and female animals. Forty (13%) of the three faecal samples were identified as positive *Listeria* species. Out of the forty positive samples, 15(37.5%) were from cattle, 16(40%) from sheep and 9(22.5%) were from goats, the difference was not statistically significant (P > 0.05). The sex distribution of the animals positive for *Listeria* species showed that 21(7%) of the positive samples were from males and 19 (6%) were from females. The sex specific prevalence in the animal species sampled was not statistically significant (P > 0.05). Biochemical characterisation of the *Listeria* isolates showed *Listeria monocytogenes* 4(10%), *Listeria innocua* 17 (42.5%), *Listeria ivanovii* 12 (30%), *Listeria seeligeri*4(10%), and *Listeria welshimeri* 3(7.5%). This study affirms the isolation of *Listeria* species in the faeces of ruminants brought for slaughter at the abattoir which could serve as a source of contamination of meat meant for human consumption.

Key words: Listeria species, ruminants, north-eastern Nigeria.

INTRODUCTION

The genus *Listeria* represents a group of closely related, Gram-positive, facultative anaerobic, non-spore-forming, rod-shaped bacteria 0.5 µm in width and 1–1.5 µm in length, and with a low G+C content. There are a total of 7 species of *Listeria* as *Listeria monocytogenes, Listeria innocua, Listeria welshimeri, Listeria seeligeri, Listeria ivanovii, Listeria murray*iand *Listeria grayi* (Gebretsadik et al., 2011). *L. monocytogenes* and *L. ivanovii* are pathogenic (Liu et al*.,* 2006). While *L. monocytogenes* infects both man and animals, *L. ivanovii* is principally an animal pathogen that rarely occurs in man (Low and Donachie, 1997). Sporadic human infections due to *L. seeligeri* and *L. innocua* have also been reported (Perrin et al*.,* 2003). The organism is ubiquitous in nature often found in animal products such as raw milk and raw meat due to unsanitary practices during milking and slaughtering (Schuchat et al*.,* 1991). Prevalence of *Listeria* species from milk, meat, vegetables, faeces and environmental samples have been reported by several authors (Ikeh et al., 2010; Atil et al*.,* 2011; Yakubu et al., 2012; Abay et al., 2012; Brian et al*.,* 2012). The presence of *Listeria* specie in faeces was associated with the prevalence of these

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bacteria in feed (Buncic, 1991; Sanaa et al., 1993). *L. innocua*exists in the environment and in animal intestines quite commonly as such it was reported to be more commonly found in food than other *Listeria* species (Erol and Sireli, 1999). Previous studies reported that *Listeria* specie was found between 0.5 and 67% of the isolation rate in cattle and sheep feces (Skovgaard and Mogen, 1988; Husu, 1990; Vilar et al., 2007). Listeriosis is of major veterinary importance in cattle, sheep, and goats (Low and Donache, 1997), not only due to significant economical losses in livestock production through morbidity and high mortality but also with regard to food safety and public health representing a possible link between the environment and human infection. There are paucity of information on the prevalence of *Listeria* species in ruminants in Maiduguri. The present study therefore aimed at characterisation and determination of the prevalence of *Listeria* species in ruminants (cattle, sheep and goats) in Maiduguri, north-eastern Nigeria.

MATERIALS AND METHODS

Study area

The study area (Maiduguri) is located in the arid zone of Borno State with an area of about 69,436 km^2 and lies within latitude 10-13°N and longitude 12-15°E. It lies within the savannah with low records of rainfall. The area falls in the tropical continental north with dry season of between 4 to 8 months, starting from October to May followed by a short rainy season from late June to early October. The state is located within the North Eastern corner of Nigeria and has boundaries with Chad to the North East, Cameroon to the East and Adamawa State to the South West. According to the 2005 census, the population is estimated to be 4,558,668 and ranked 12th in the country.

Sample collection

A total of three hundred (300) fecal samples were aseptically collected from Maiduguri central abattoir between January -March, 2011 from apparently healthy goats, sheep and cattle. Equal numbers of samples were collected from the animals, cattle (100), goat (100) and sheep (100) comprising fifty (50) samples each from male and female animals. The fecal samples were collected per rectum after proper restraint and put into a sterile sample bottle and transported to the Faculty of Veterinary Medicine Microbiology Laboratory in an ice pack for bacteriological analysis.

Bacteria isolation

Aseptically, 10 g of each sample was added to 90 ml *Listeria* enrichment broth (LEB) (Oxoid®) containing selective *Listeria* enrichment supplement in a bottle; this was homogenized for 2 min at room temperature and incubated at 37°C for 24-48 h. After incubation, a loopful of growth from LEB was streaked onto the surface of *Listeria* selective agar (Oxoid ®) and PALCAM agar. The plates were incubated at 37°C for 24 to 48 h. Typical *Listeria* colonies appeared greyish-black with a black zone in surrounding medium of both plates. This black colour was due to the utilization of esculin in the media. Presumptive colonies of *Listeria* species on both plates having a black colouration on PALCAM and LSA were streaked on nutrient agar slants incubated at 37°C for 24 h and stored at 4°C.

Presumptive colonies were subsequently subjected to Gram staining and further biochemical characterisation. Biochemical characterization was conducted using beta haemolytic reactions, catalase, oxidase, urease and acid production from (glucose, manitol, galactose, xylose and rhamnose) in order to differentiate the various *Listeria* species according to the methods of OIE (2008) manual.

Statistical analysis

The data collected was subjected to Fisher's Exact Chi-square test and odds ratio using Graph PadInstat statistical package to determine if there is significant association between sex and isolation of *Listeria* species in ruminants in the study area. A P value less than 0.05 was considered statistically significant. The prevalence rate and the odds ratio (OR) were calculated using (2x2) contingency table to test for association between isolation of *Listeria* species in the faeces and sex as well as animal species (cattle, sheep, goats).

RESULTS

A total of three hundred (300) faecal samples comprising of 100 samples each from cattle, sheep and goats were collected at the Maiduguri central abattoir and analysed for the presence of *Listeria* species. Forty (13%) out of the three hundred samples were presumptively identified as positive for *Listeria* species, while the remaining 260 (87%) samples were found to be negative for *Listeria* species. Out of the 40 positive samples, 21 (7%) were male (OR = 0.576 – 2.186) and 19 (6%) were female (OR = 0.457 – 1.735). There was no significant statistical association (P > 0.05) between male and female positive animals as regards to isolation of *Listeria* species (Table 1).

Table 2 shows the sex specific prevalence of *Listeria* species in cattle, sheep and goats in Maiduguri. *Listeria* species were isolated in fifteen (15%) out of the one hundred faecal samples collected from cattle, this consist of 7% males (OR = 0.284 – 2.568) and 8% females (OR = 0.389 – 3.516) which was not statistically significant (P > 0.05). Out of the hundred faecal samples collected from sheep,16% were positive for *Listeria* species comprising of 8% male and 8% female with the same (OR = 0.343 – 2.914) having no statistical difference (P > 0.05) between both sexes. A total of 9% out of the hundred faecal samples examined from goats were positive for *Listeria* species, out of which 6% were males (OR = 0.503 – 9.017) and 3% were females (OR = 0.110 – 1.987). There was also no statistically significant association (P > 0.05) between the sex of the animals and the isolation *Listeria* species.

The source specific prevalence of *Listeria* species in ruminants is shown in Table 3. Out of the fifteen cattle from which *Listeria* species were isolated, six each were *L. ivanovii* and *L. innocua* and one each were *L. monocytogenes*, *L. seelighreii* and *L. welshimeri*. The isolation rate of *Listeria* species in sheep was sixteen, these comprises 8 positive samples for *L. innocua*, 4 for *L. ivanovii* and 2 each for *L. monocytogenes* and *L. seeligheri*. *Listeria* species were isolated in 9 goats in the present study. These consist of 3 positive samples for *L. innocua*, 2 each for *L. ivanovii* and *L. welshimeri* and 1 positive sample each for *L. monocytogenes* and *L. seeligheri*.

Table 1. Sex specific prevalence of *Listeria* species in faeces of ruminants sampled in Maiduguri central abattoir (January – March 2011).

Sex	Positive	Negative	Total	X2	Odds ratio (OR)	95% CI on OR	
						Lower	Higher
Male	21(7%)	129(44%)	150(50%)	0.115*	0.122	0.576	2.186
Female	19(6%)	131(43%)	150(50%)		0.891	0.457	1.735
Total	40(13%)	260(87%)	300(100%)				

*The difference in the sex prevalence was not statistically significant (P > 0.05).

Table 2. Sex specific prevalence of *Listeria* species in faeces of cattle, sheep and goats in Maiduguri central abattoir (January – March 2011).

Animal	Sex	Positive	Negative	Total	X^2	Odds ratio (OR)	95% CI on OR	
							Lower	Upper
	Male	7	43	50		0.855	0.284	2.568
Cattle	Female	8	42	50	0.079*	1.170	0.389	3.516
	Total	15	85	100				
	Male	8	42	50		1.00	0.343	2.914
Sheep	Female	8	42	50	0.00*	1.00	0.343	2.914
	Total	16	84	100				
	Male	6	44	50		2.136	0.503	9.017
Goats	Female	3	47	50	1.099*	0.468	0.110	1.987
	Total	9	91	100				

*****The sex specific prevalence among the animal species was not statistically significant (P>0.05).

Table 3. Source specicific prevalence of *Listeria* species in faeces of cattle, sheep and goats in Maiduguri central abattoir (January – March 2011).

Animal specie	Total no. of isolates	*Listeria* species				
		L. monocytogenes	*L. ivanovii*	*L. seeligeri*	*L. innocua*	*L. welshimeri*
Cattle	15	1	6	1	6	1
Sheep	16	2	4	2	8	-
Goat	9	1	2	1	3	2
Total	40	4	12	4	17	3

DISCUSSION

An isolation rate of 13% *Listeria* species in the faeces of ruminants have been reported in the present study which is similar to the reports of Abay et al*.* (2012) who also reported the isolation rate of 22 and 10% of *Listeria* species in the faeces of cattle and sheep, respectively. The variation in the isolation rate could be due to the number of animals sampled and the difference in geographical locations or the sampling technique employed. In the present study, phenotypic methods of characterisation were employed using 300 animals comprising 100 each of cattle, sheep and goats. Other workers in different countries (Choi et al*.* 2001) in Korea, Miettinen et al. (2001) in Finland and Hassan et al*.* (2001) in Malaysia) have reported an incidence of between 62 and 85% of *Listeria* species in various foods. The findings here are similar to those of MacGowan et al*.* (1994) that the usual habitat of *Listeria species* is the intestinal tract of mammals and birds from where the organism enters the soil via animal droppings. From the results of the present study, more male animals (21 (7%)) were positive for *Listeria* species than female animals (19 (6%)). The difference in the sex specific prevalence was not statistically significant (P > 0.05). In cattle, more female cattle (8(8%)) are affected than male cattle (7(7%)) but the diffe-

rence was not significant statistically (P > 0.05). There was no difference in the number of male and female sheep that were positive for *Listeria i*n the present study. In the goats sampled in the present study, more male goats (6(6%)) were positive for *Listeria* species than females (3(3%)) but the difference was not statistically significant (P > 0.05).

The findings in the present study has affirmed the isolation of *Listeria* species in ruminants which may serve as reservoir for human pathogenic strains and therefore its impact on food safety cannot be over-emphasised (Borucki et al*.,* 2004; Nightingale et al*.,* 2004; Okwumabua et al*.,* 2006). Animals can carry the bacterium without appearing ill and can contaminate foods of animal origin such as meats and dairy products (Schuchat et al*.,* 1992; Hood, 1993; Bockserman, 2000). Although the portal of entry of *L. ivanovii* has not been fully established, *L. ivanovii* infection in ruminants is associated with eating spoiled silage or hay, as happens with *L. monocytogenes*, suggesting foodborne origin (Gaya et al*.,* 1996).

Ruminant farm animals play a key role in the persistence of *Listeria* spp. in the rural environment via a continuous faecal-oral cycle (Vazquez-Boland et al., 2001). In the present study, the majority of *Listeria* species isolated were *L. innocua* as it has been reported to be present in much larger numbers in feed than other species, and therefore has a higher chance of being detected in animal faeces. It has been reported that *L. innocua* isolated from beef minced meat and other *L. innocua* isolated from cattle faeces have 99% similarity (Abay et al*.,* 2012). Hence *Listeria* isolated in this study (faeces) could be considered as a potential risk for meat contamination that could play a role in the epidemiology of listeriosis.

*L. ivanovii*is considered to be mildly pathogenic and seems to affect almost exclusively ruminants, resulting in abortion, still-births, and neonatal septicemia, but not central nervous system infection (Low and Donachie, 1997; McLauchlin and Jones, 1999; V'azquez-Boland et al*.,* 2001). The association between the isolated pathogens and silage consumed by the ruminants could not be ascertained because no sample was collected from the silage and the type of the feed consumed by these ruminant were not collected for microbiological analysis, though it has been reported that investigation of an epidemiological link between silage feeding and listeriosis in ruminants gave inconsistent results. Whilst some studies could isolate matching *Listeria* strains in brains of affected animals and silage samples, others yielded unrelated strains (Mohammed et al*.,* 2009). Previous study reported a higher prevalence of the bacterium in samples collected from the immediate cattle environment (feed bunks, water through and beddings) and in cattle feces than in silage challenging the view that silage is the only source of Listeriosis (Mohammed et al*.,* 2009).

Conclusion and recommendations

The study established the findings that *Listeria* species are found in the faeces of ruminants (13%) in the study area. *Listeria* species were more commonly isolated in cattle (15%) and sheep (16%) than in goats (9%). *L. innocua*17 (42.5%) and *L. ivanovii*12 (30%) were the *Listeria* species more commonly isolated in the faeces of ruminants than the other species. We therefore recommend the use of good hygienic practices and standard procedures in the abattoir to minimise the level of contamination of meat.

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Full Length Research Paper

Biochemical characterization of indigenous Fulani and Yoruba ecotypes chicken of Nigeria

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The study was carried out to characterize two indigenous chickens of Nigeria using protein markers; haemolglobin (HB) and carbonic anhydrase (CA). Separation of the two proteins was achieved by cellulose acetate electrophoresis and direct gene counting method was employed to interpret the result. Palentological statistics was used to generate dendrogram that measured genetic similarity within and between each of the population studied. HB was interpreted into three phenotypes: AA, AB and BB which were genetically controlled two codominant alleles HB^A and HB^B. Allele frequencies of HB^A and HB^B in Yoruba ecotype chicken were 0.34 and 0.66, respectively, while those of Fulani ecotype were 0.28 and 0.72, respectively. CA was also interpreted into three phenotypes (FF, FS and SS) which are genetically controlled by two codominant alleles CA^F and CA^S; their respective allele frequencies were 0.33 and 0.67 in Yoruba ecotype chicken and 0.24 and 0.76 in Fulani ecotype chicken. Genetic similarity within ecotype indicated 60% in Fulani, 80% in Yoruba and 40% between Yoruba and Fulani at HB locus while at CA locus, genetic similarity was 69% in Fulani ecotype, 50% in Yoruba ecotype and 42% between Yoruba and Fulani ecotype. Cavalli-Sforza genetic distance between the two Ecotypes was $2.1x10^{-2}$. Conclusively, the two populations were genetically related and further studies should focus on other protein markers and at molecular level.

Key words: Ecotype, protein marker, genetic distance and selection.

INTRODUCTION

There is a great concern globally over the loss of biodiversity in domestic animal and plants. Part of the Nigerian heritage lies in the genetic diversity of native breed. Very limited information on these populations concerning genetic diversity exists. There is a major global thrust on genetic preservation and biodiversity which is reflected in efforts on the development of the genome data banks (Crawford and Gavora, 1993). These initiatives have come at an opportune time, because of continued uncontrolled breeding practices among indigenous chicken which do not that consider gene preservation aspects would lead to the erosion of native germplasm (Bessei, 1989). However, little has been done to characterize and conserve the indigenous chicken genetic resources of Nigeria. Unfortunately, like in other developing countries, attention is directed to commercialization using improved breeds. However, not enough attempts have been made to evaluate the genetic characteristics and consequently improve the Nigerian indigenous chickens.

The conservation of domestic animal diversity is essential to meet future needs in Nigeria and Africa as a whole. In order to cope with an unpredictable future, genetic reserves capable of readily responding to directional forces

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imposed by a broad spectrum environment must be maintained. Maintaining genetic diversity is an insurance package against future adverse conditions (FAO, 2000b). Due to diversity among environments, nutritional standards and challenges from infectious agent, a variety of breeds and population are required. These will act as storehouses of genetic variation which will form the basis for selection and may be drawn upon in times of biological stress such as famine, drought or disease epidemics.

In addition to increasing global human population pressures, the quantity of food and other products must increase. Not only should diversity be maintained for practical purposes, but also for cultural reasons. A community's domestic animals can enhance the environment as a living system, thus also enhancing the human inhabitant's quality of life. The need for characterization comes from the potential rate of decrease of genetic variation. The loss of genetic variation within and between breeds is detrimental not only from the perspectives of culture and conservation but also utility since lost genes may be of future economic importance (FAO, 2000b). Within breed, high rates of loss of genetic variation leads to reduced chances of breed survival due to decreased fitness through inbreeding depression. These breeds become subject to faster changes in gene frequencies, greater rate of loss of genes and genetic constitutions. These are all due to small effective population sizes, or, equivalently, high rates of inbreeding (Meuwissen et al., 2001).Once animal genetic diversity has been lost, it cannot be replaced. Advances in biotechnology offer possibilities of improving, utilizing and characterizing present domestic animal diversity. Characterization at the biochemical and morphological level offer the opportunity to explore genetic diversity within and between livestock populations and to determine genetic relationships among populations and that the method is rapid, relatively affordable and reliable. The present study is therefore designed to characterize two ecotype of indigenous chickens through biochemical markers.

MATERIALS AND METHODS

The experiment was conducted at the Animal Breeding and Genetics laboratory section of the Department of Animal Science, University of Ibadan. Ibadan is located on the latitude 7°20'N and longitude 3°51 E, 200 m above the sea level.

Blood samples were collected through wing veins from 100 chickens comprising 50 adult Yoruba ecotype and 50 Fulani ecotype, placed in heparinized tubes to prevent coagulation and were refrigerated. Samples were prepared and subjected to cellulose acetate electrophoresis following the procedure of Riken (2006).

Sample preparation

Red cells

Blood samples (5 ml) collected were transferred from wing veins of the individual chickens into plastic tubes containing anticoagulant. Red blood cells (RBC_S) were prepared from the erythrocyte fraction of heparinized blood by centrifuging at 2500-3000 rpm for 10 min at 4°C. The RBCs were washed in saline buffer three times by repeating centrifugation at 2,500-3000 rpm for 5 min at 4°C. The RBCs were lysed with eight fold volumes of water. The red cell lysates were stored for further analysis of haemolglobin (HB) and carbonic anhydrase (CA).

Cellulose acetate electrophoresis protocol

The cellulose acetate membrane was soaked very slowly in the buffer solution for over 5 min. Buffer corresponding to each of the protein was poured into the electrophoresis chamber. Wicks were folded and moistened with appropriate buffer and placed on each of the support arm of the electrophoresis chamber. The samples were poured into the slots of the applicator. The soaked cellulose acetate membrane plate was gently placed between the paper towels on each support arms of the electrophoresis chamber. The comb was stamped into the applicator and placed on the gel upside down on the paper rows inside the electrophoresis chamber. Coin was placed on the gel to keep plate flat and ensured an even current distribution through the plate. The electrophoresis was run as described below for each of the protein.

Haemoglobin (HB)

For haemoglobin the electrophoresis included: tissue sample, RBCs in 8 volumes of H_2O; buffer system, Tris EDTA borate; pH 8.4; supporting media, cellulose acetate membrane; electrophoresis, voltage of 350 V; time of 40 min; temperature of 4°C; migration cathode (-) to anode (+); stain procedure, staining with Ponceau S and destaining in 5% acetic acid.

Carbonic anhydrase (CA)

For carbonic anhydrase, the electrophoresis included: tissue sample, RBCs in 4 volumes of H_20; buffer system: -EDTA sodium acetate; pH 5.6; supporting media, cellulose acetate membrane; electrophoresis voltage of 200 V; time of 45 min; temperature of 4°C; migration, anode (+) to cathode (-); stain procedure, staining with ponceau S and destaining in 1% acetic acid.

Temperature of 4°C was achieved by placing the electrophoresis chamber inside the refrigerator.

After destaining, the bands were clearly separated and direct allele counting method was used for each of the protein as follows: A single faster band was designated as the AA for HB and FF for CA homozygous. The presence of a single slower band was designated as BB for HB and SS for CA homozygous. The presence of both bands was designated AB for HB and FS for CA heterozygous.

Statistical analysis

Gene frequency was calculated using the expression provided by Roghgarden (1977) as follows:
Let P= Gene frequency of allele x; Q= gene frequency of allele y.

$$P = \frac{2(Nxx) + Nxy}{2N}$$

$$Q = \frac{2\,(Nyy) + Nxy}{2\,N}$$

Where, N is the total number of individual sampled; N_{xx} is the observed genotype number for xx; N_{xy} is the observed genotype

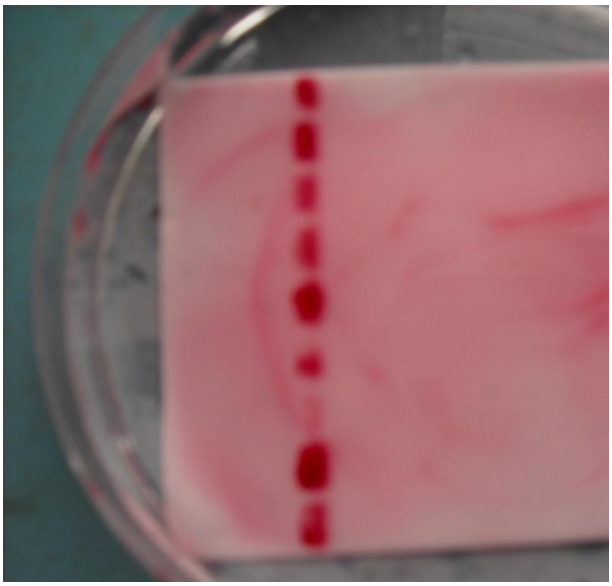

Plate 1. Electrophoretic separation of haemoglobin in fulani ecotype chickens.

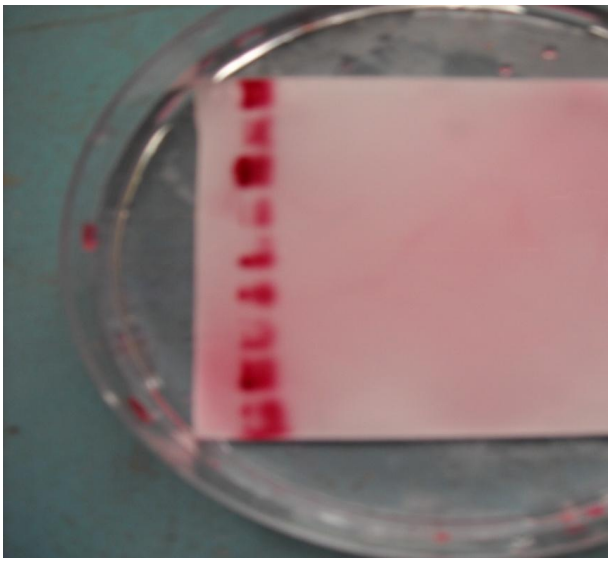

Plate 2. Electrophoretic separation of haemoglobin in yoruba ecotype chickens.

number for xy; N_{yy} is the observed genotype number for yy. Genotype frequency was calculated as follows: (Number of xx / Total individual) x 100; (Number of xy / Total individual) x 100; (Number of yy/ Total individual) x 100. Paleontological Statistical (PAST) package was used to generate dendogram that measure genetic similarity.

Estimation of genetic distance

The simplest measure of genetic distance as proposed by Nei (1972) and Cavalli-Sforza (1967) called minimum genetic distance (DM) was used.

Plate 3. Electrophoretic separation of carbonic anhydrase in fulani ecotype chickens.

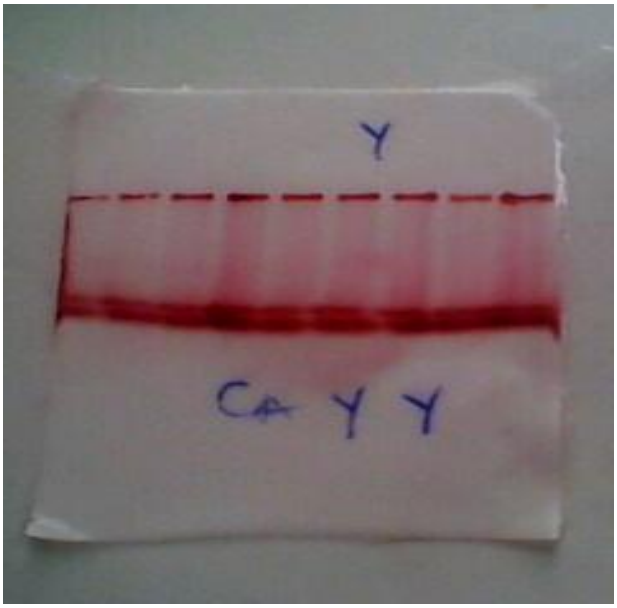

Plate 4. Electrophoretic separation of carbonic anhydrase in yoruba ecotype chickens.

RESULTS

Biochemical polymorphism

Electrophoretic separation of HB and CA are shown in Plates 1 to 4. HB was interpreted into three phenotypes (AA, AB and BB) which were genetically controlled by two codominant alleles (HB^A and HB^B). The allele frequencies (Table 1) of Hb A and Hb B in Yoruba ecotype chicken were 0.34 and 0.66 while those of Fulani ecotype chicken were 0.28 and 0.72, respectively. The genotype frequencies

Table 1. Allele and allelic frequencies at haemoglobin locus with respect to ecotype.

Ecotype	Number	Allelic frequency							
		AA	%	AB	%	BB	%	A	B
Yoruba	40	5	12.5	17	42.5	18	45	0.34	0.66
Fulani	36	6	16.67	8	22.22	22	61.11	0.277	0.722

Table 2. Allele and allelic frequencies at carbonic anhydrase locus with respect to ecotype.

Ecotype	Allele frequency								
	Number	FF	%	FS	%	SS	%	F	S
Yoruba	40	6	15	14	35	20	50	0.325	0.675
Fulani	36	3	8.33	11	30.55	22	61.11	0.236	0.763

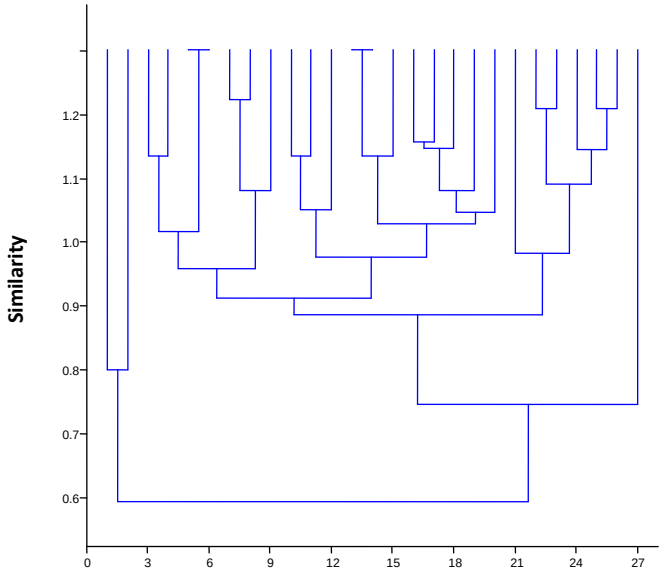

Figure 1. Dendogram of genetic similarity of fulani ecotype chicken at HB locus.

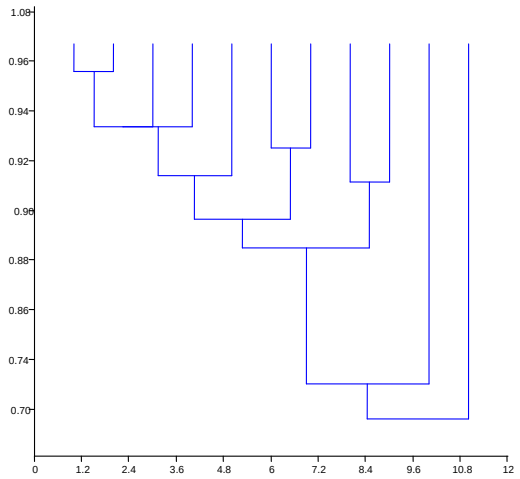

Figure 2. Dendogram of genetic similarity of Fulani ecotype chicken at CA locus.

of 12.5, 42.5 and 45% were recorded for AA, AB, BB, respectively, for Yoruba ecotype chickens while Fulani ecotype chickens had 16.67, 22.2 and 61.11%, respectively, for AA, AB and BB. HB^A was lower than HB^B in the two populations. The distribution of allele and allelic frequencies of CA (Table 2) is comparable to those obtained with HB and it was interpreted into three phenoltypes (FF, FS and SS) which are genetically controlled by two codominant allele (CA^F and CA^S). Their respective allele frequencies were 0.33 and 0.67 in Yoruba ecotype chicken and 0.24 and 0.76 in Fulani ecotype chicken, respectively. Genotype frequencies were 15, 35 and 50% in Yoruba ecotype and 8.33, 30.55 and 61.11% in Fulani ecotype for FF, FS and SS, respectively. Generally allele frequency of F allele was low in the two populations studied compared to S loci allele.

Figures 1-4 represent dendrograms that measured the genetic similarity within and between the two populations studied herein. In Fulani ecotype, three clusters were observed for HB (Figure 1); one main cluster (60%) and two sub clusters (75%) and two major clusters (69 and 69%) were observed at CA locus (Figure 2). Generally, the genetic similarity is high within the population. In Yoruba ecotype, two major clusters locus (80%, 62%) were observed at HB (Figure 3), while one major cluster (50%) was observed at CA locus (Figure 4) with two sub cluster (56 and 60%). High genetic similarity was also noticed at all the loci within the population. Dendrogram showing genetic similarity between the two populations studied (Yoruba and Fulani ecotype) at HB and CA loci are represented in Figures 5 and 6, respectively. One major cluster (40%) and two sub clusters were observed at HB locus. Similarly, one major cluster (42%) and two sub clusters were observed at CA locus. Also, genetic distance as indicated by Carvalli-Sforza (2.1×10^{-2}) is low which equally indicate high genetic similarity.

DISCUSSION

The biochemical markers have been extensively utilized

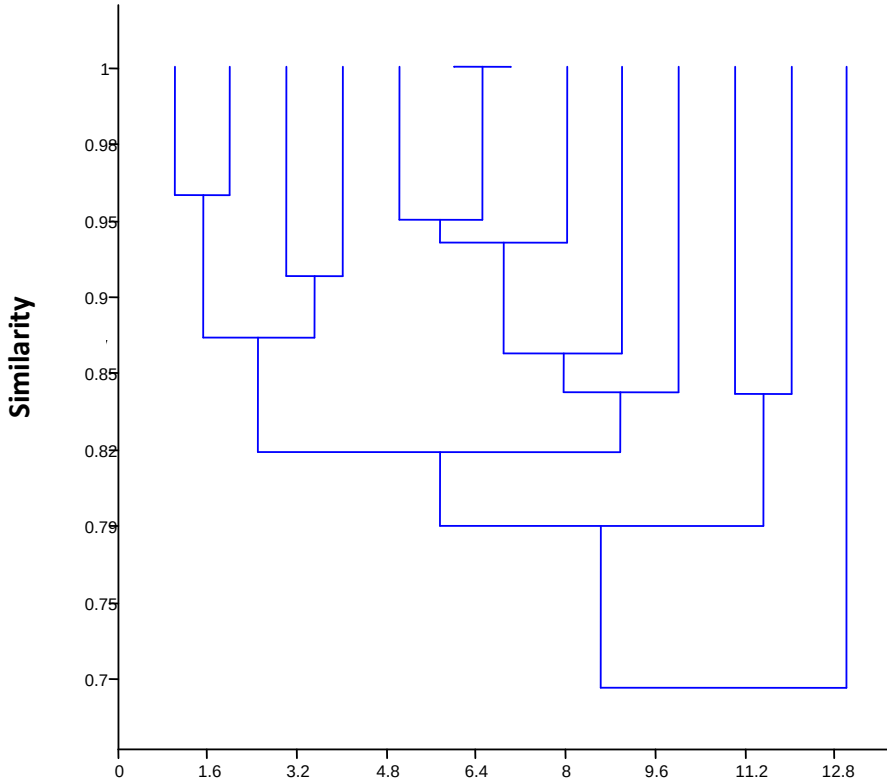

Figure 3. Dendogram of genetic similarity of Yoruba ecotype chicken at HB locus.

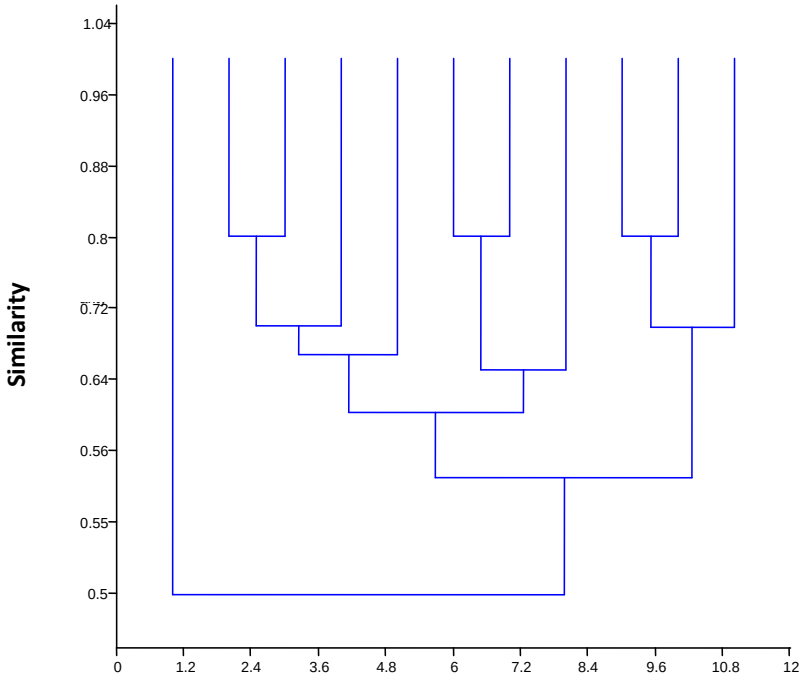

Figure 4. Dendogram of genetic similarity of Yoruba ecotype chicken at CA locus.

for documenting genetic similarities or diversities of different populations of livestock comprising a species, a strain or even closely related line (Lee et al., 2000; Esmaeilkhanian et al., 2000; Zhang et al., 2002 ; Salako

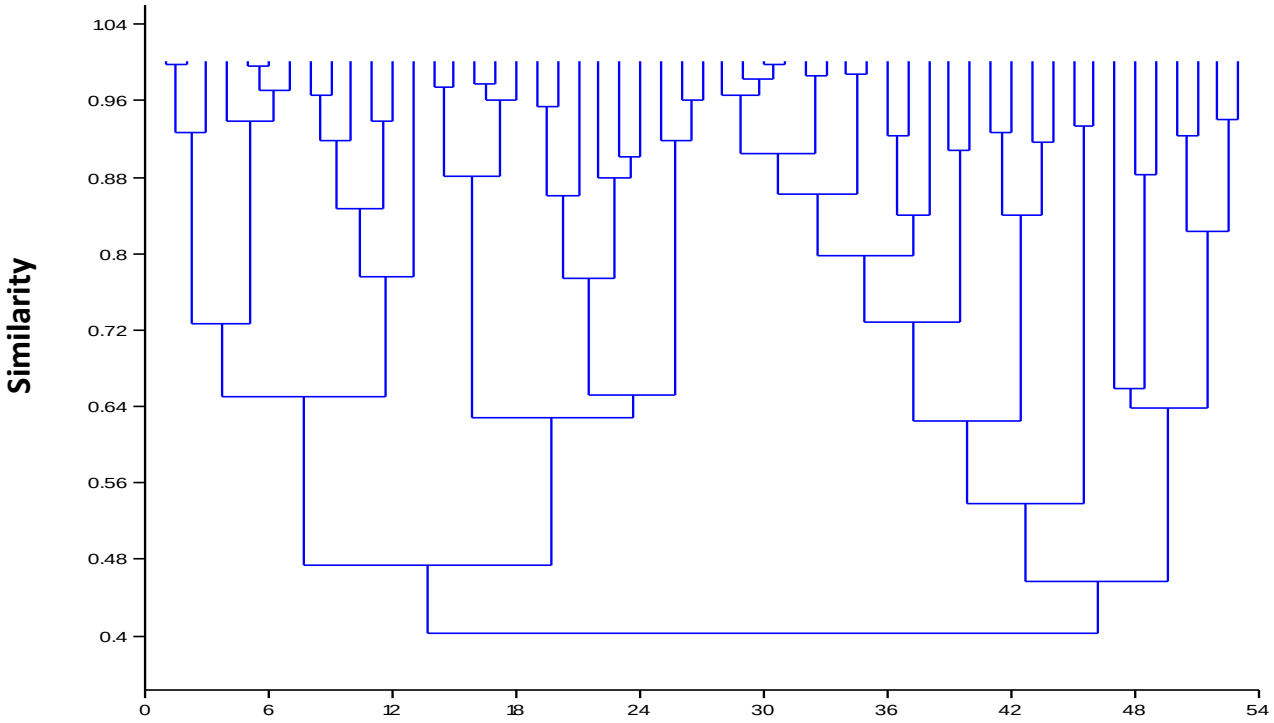

Figure 5. Dendogram of genetic similarity between Yoruba and Fulani Ecotype Chicken at HB locus.

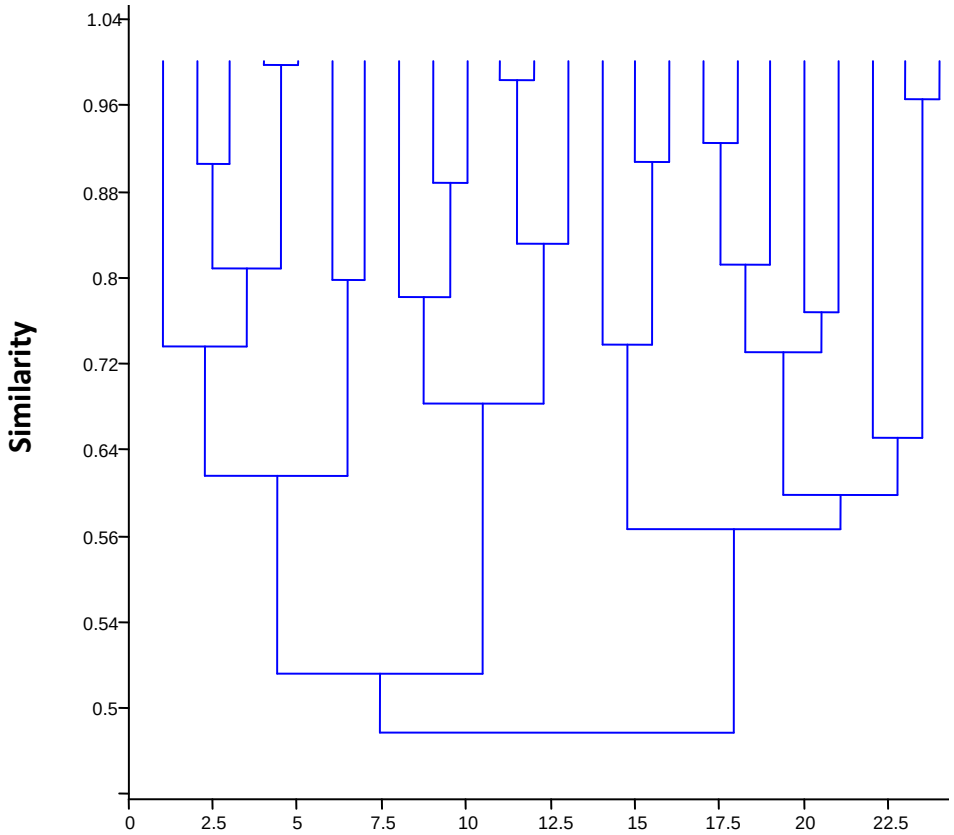

Figure 6. Dendogram of genetic similarity between Yoruba and Fulani Ecotype Chicken at CA locus.

and Ige, 2006 ; Dimri, 1981 ; Mazumder et al., 1989; Washburn et al., 1971 ; Yamamoto et al., 1996). According to Dimiri (1981), three types of haemoglobin were observed (AA, AB and BB) and which were controlled by two autosomal alleles A and B. Similar result was observed in the two populations studied. Mazumder et al. (1989) reported frequencies of 0.96 (HB^A) and 0.04 (HB^B) for white leghorn chickens, and 1.00 (HB^A) for broiler which contradicted the result of this work as frequency of HB^A were 0.34 and 0.28 in Yoruba and Fulani ecotype chickens, respectively, while frequency of HB^B were 0.66 and 0.72, respectively. Frequency of Hb^B was predominant in both populations. Mazumder et al. (1989) reported the presence of gene fixation as only genotype HB^{AA} was identified in their study. However, the discrepancy observed was primarily attributed to the specific genetic background of the breeds. Singh and Nordskog (1981) also found complete gene fixation for haemoglobin in inbred line chickens. Lee et al. (2000) also reported that Korea native chicken were monomorphic at haemoglobin locus. Salako and Ige (2006) reported frequencies of 0.68 (HB^A) and 0.33 (HB^B) in a mixed population of indigenous chickens of Nigeria. Washburn et al. (1971) related haemoglobin types with Marek disease and concluded that chickens with homozygous mutant haemoglobin genotypes were approximately 20% less susceptible to Marek disease. In the same way, Dimri (1981) reported that haemoglobin polymorphism affects growth rate and hatchability, with the highest in AA (62.20%) followed by HB AB (48.20%) and BB (31.50%). The transport of CO_2, haemoglobin utilization for controlling pH of body fluids and selection for the production of carbonate ions are facilitated by carbonic anhydrase. Frequency of Ca^F was higher than Ca^S in both Fulani and Yoruba ecotype population; this observation suggest a close relationship between the two populations. There is no available information in literature on carbonic anhydrase types in chicken, however, it has been reported extensively in other livestock animals. Also, activity of CA has been positively correlated with egg shell thickness.

Genetic differences between breeds, ecotypes and populations are controlled by mutation, genetic drift, selection and migration (Eding and Laval, 1999). Therefore, the evaluation of indigenous chicken population as genetic resources includes the determinations of genetic distance between the available populations (Hamnond, 1994). The genetic distance between the two ecotypes as measured by Cavalli-Sforza was 2.10×10^{-2} which is quite low indicating little genetic effect of drift or mutation. It also reflected that these populations are not genetically isolated from each other. Kaya and Yildiz (2008) reported similar findings among Turkish native chickens. They estimated genetic distance to be 6.5×10^{-2} between the populations. Hillel et al. (2003) reported higher value of 0.44 using Nei's mean genetic distance between given populations using microsatellite markers. These findings also imply high levels of genetic flow among the ecotypes resulting in admixed populations. Genetic similarity as measured by dendogram equally supported high genetic flow between two ecotypes.

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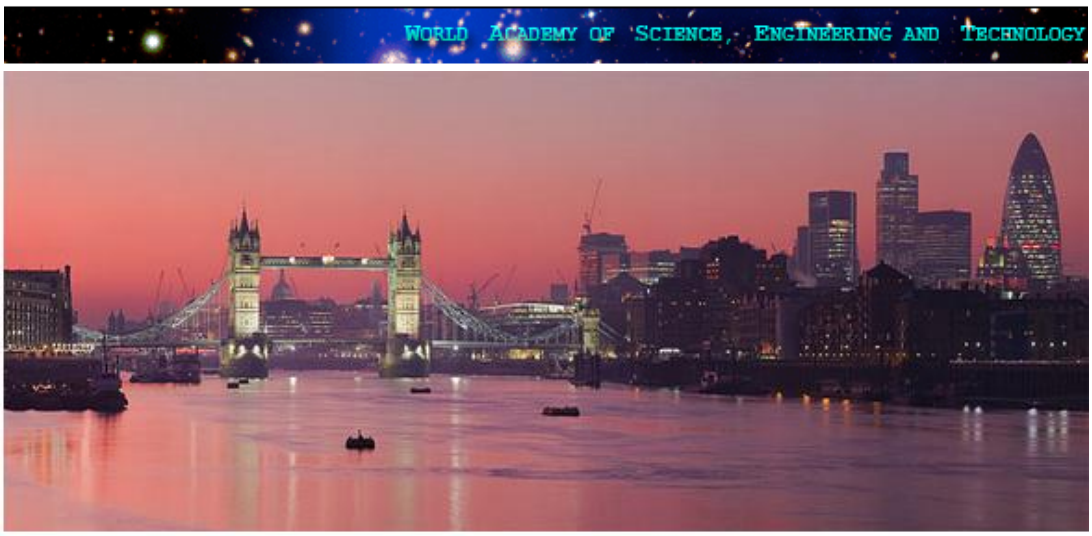

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