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Full Length Research Paper

Genetic characterization of fin fish species from the Warri River at Ubeji, Niger Delta, Nigeria

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A study to evaluate the genetic similarities and differences among 11 specimens of cichlids and four specimens of mudcatfishes obtained from Warri River was carried out through DNA fingerprinting analysis using random amplified polymorphic DNA (RAPD)-PCR amplification with seven decamer primers and dendrograms through unweighted pair-group method with average (UPGMA) cluster analysis. The total number of bands generated by the seven RAPD primers, ranged between 2 to 33 for the cichlids and 8 to 28 for the catfish family, with band size between 100 to 800 bp. The primers produced 228 bands in total 119 for the cichlids and 109 for the catfishes, with 24% polymorphism. Considerable genetic variation was observed within species (especially within *Tilapia zilli, T. guineensis* and *Clarias gariepinus*), between species in the same genera (*T. zilli and T. guineensis)* and among cichlids and catfishes. The most consistent of the RAPD primers generated 87 bands among the cichlids with 23 bands (26%) polymorphic and 74% conserved. Among the catfishes, the primers produced 69 bands with 16 (23%) polymorphic. The data show that the RAPD technique was useful and sensitive in differentiating various fish genera and species.

Key words: Random amplified polymorphic DNA (RAPD), Niger Delta, aquatic diversity, phylogeny

INTRODUCTION

There is an increase in research interest in the maintenance and preservation of genetic diversity of fish as an essential ecological resource and life support system (Ekelemu and Zelibe, 2006). Fishes constitute a large percentage of the biological diversity of organisms that inhabit rivers and other water bodies and majority of the population of Niger Delta depends on catch from the wild as source of animal protein. The mudcatfishes and the tilapias are some of the groups of fishes that are frequently caught (Idodo-Umeh, 2003; Akpan, 2013).

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Therefore, scientific efforts to determine the current level of diversity of common fishes in these localities are applicable in ecology, fishery management, aquaculture and stock conservation and in resolving perennial identification problems among cichlids and mudcatfish species.

Traditionally, morphological characterization of fish has been useful in determining fish species, sex and larval stages, but it does not offer a definite or reliable method for species identification. Hence, genetic or DNA-based techniques are being used for such purposes (Teletchea, 2009; Wong, 2011). Species identification has been shown to be possible using molecular markers in Random Amplified Polymorphic DNA (RAPD) (Soufy et al., 2009). The RAPD technique is a polymerase chain reaction (PCR) technique that relies on the generation of amplification products for a given nucleic acid using an amplification-based scanning technique driven by arbitrary priming oligonucleotides (Williams et al., 1990). The amplified region consists mostly of non-coding and unstructured sequences that are of different length from one species to another (Williams et al., 1990). In this technique, polymorphism is revealed by the presence or absence of amplification products when two strains or individuals are compared (Soufy et al., 2009). High levels of DNA polymorphisms could be detected using this technique; it could produce fine genetic markers, and a large number of loci sampled without prior DNA sequence information (Ali et al., 2004; Ahmed et al., 2004; Theodorakis and Bickham, 2004).

The RAPD technique has been used in the evaluation and identification of the genetic diversities of species and subspecies of tilapia (Bardakci and Skibinski, 1994; Dinesh et al., 1996; Ahmed et al., 2004; Ali et al., 2004; Sherif et al., 2009; Soufy et al., 2009); carps (El-Zaeem et al., 2006); *Clarias gariepinus* (Saad, et al., 2009); *Prochilodus marggravii* (Hatanaka and Galetti, 2003); *Pimelodus maculatus, P. lineatus, Salminus brasiliensis and Steindachneridion scripta*, (Ramella et al., 2006). Similarly, Hassanien et al. (2004) found genetic diversity among different populations of *Oreochromis niloticus* in Egypt using this technique.

In our study, we report the usefulness of the RAPD technique in the assessment of genetic variations among species of cichlids and mud catfish from the Warri River, Niger Delta, Nigeria.

MATERIALS AND METHODS

Description of study area

The Warri River is a major navigable channel of the Niger Delta, southern Nigeria. It takes its origin from around Utagba Uno and flows through zones of freshwater swamps, mangrove swamps, dominated by *Rhizophora* species, and coastal sand ridges. It is a relatively large water body which stretches within latitudes 5°21' to 6°00' N and longitude 5°24' to 6°21'E, covering a surface area of about 255 sq km with a length of about 150 km. It drains various tributaries and empties into the brackish Forcados River that in turn empties into the Atlantic Ocean at the Bight of Benin (Figure 1).

Sampling stations were established along the river at Ubeji, a remote village located behind a refinery (Figure 1). The river is known to supply drinking water for domestic use and irrigation of farm lands, recreational activities and means of transportation. Fishing activities also takes place in the river and the people depend to a large extent on their fish catches as one of the major sources of protein.

Collection of samples

Fifteen (15) fish specimens were collected from the Warri River at Ubeji for this study in June 2010 from local fishermen. The fish samples were chilled in the field, frozen and transported to the Curator, Department of Zoology University of Ibadan, where eleven were identified as cichlids (five specimens of *Tilapia zilli*, three of *T. guineensis*, one *Sarotherodon galilaeus* and two *Hemichromis fasciatus*) and four as catfishes (*C. gariepinus*). They were then stored frozen in the laboratory prior to RAPD analysis. Three individuals of *C. gariepinus* bought from the market in Ibadan served as control.

DNA extraction

DNA was extracted from the muscle tissue following the method described by Lopera-Barrero et al. (2008) with some modification. Approximately, 0.3 g of each muscle tissue was crushed with a mortar and pestle in 500 µL extraction buffer (100 mM Tris, 8.5 mM EDTA, 500 mM NaCl) and transferred to a polypropylene microfuge tube. 20% SDS was added; the mixture was vortexed briefly and incubated in a gentle shaking water bath at 65°C for 10 min. 160 µL of 5 M potassium acetate was added after bringing the sample to room temperature, then vortexed and centrifuged at 10 000 *g* for 10 min. Supernatant was removed (about 500 µL) into another Eppendorf tube and 200 µL of cold isopropanol was added and mixed after which samples were kept on at -4°C for 15 min before centrifuging at 10 000 *g* for 10 min to sediment the DNA. Supernatant was decanted after centrifuging and pellets were washed with 500 µL of 70% ethanol and centrifuged at 10 000 *g* for 10 min. The ethanol was decanted and the DNA was air-dried at room temperature (37°C) and re-suspended in 50 µL TE (10mM Tris and 1mM EDTA) buffer and stored in the freezer prior to RAPD PCR analysis. The integrity of the DNA was verified by electrophoresis on a 1% agarose gel.

RAPD PCR Analysis

The PCR consisted of a 25 µL reaction volume of 4x Go Taq (Integrated Technologies U.S.A) colourless master mix, 1 µL of 7 pmol of primer (10 bp) and 2 µL of genomic DNA (100 to 200 ng). The sequences of primers used are shown in Tables 1 and 2. The final reaction mixture was placed in a DNA thermal cycle (Apicon ThermoEX 500). The PCR programme included an initial denaturation step at 94° C for 2 min followed by 45 cycles of 94°C for 30 s for DNA denaturation annealing, extension at 72°C for 30 s and final extension at 72°C for 10 min was carried out. The samples were cooled at 4°C and the amplified DNA fragments were separated on 1.2% agarose gel and stained with ethidium bromide. The amplified DNA pattern was visualized on a UV transilluminator and photographed.

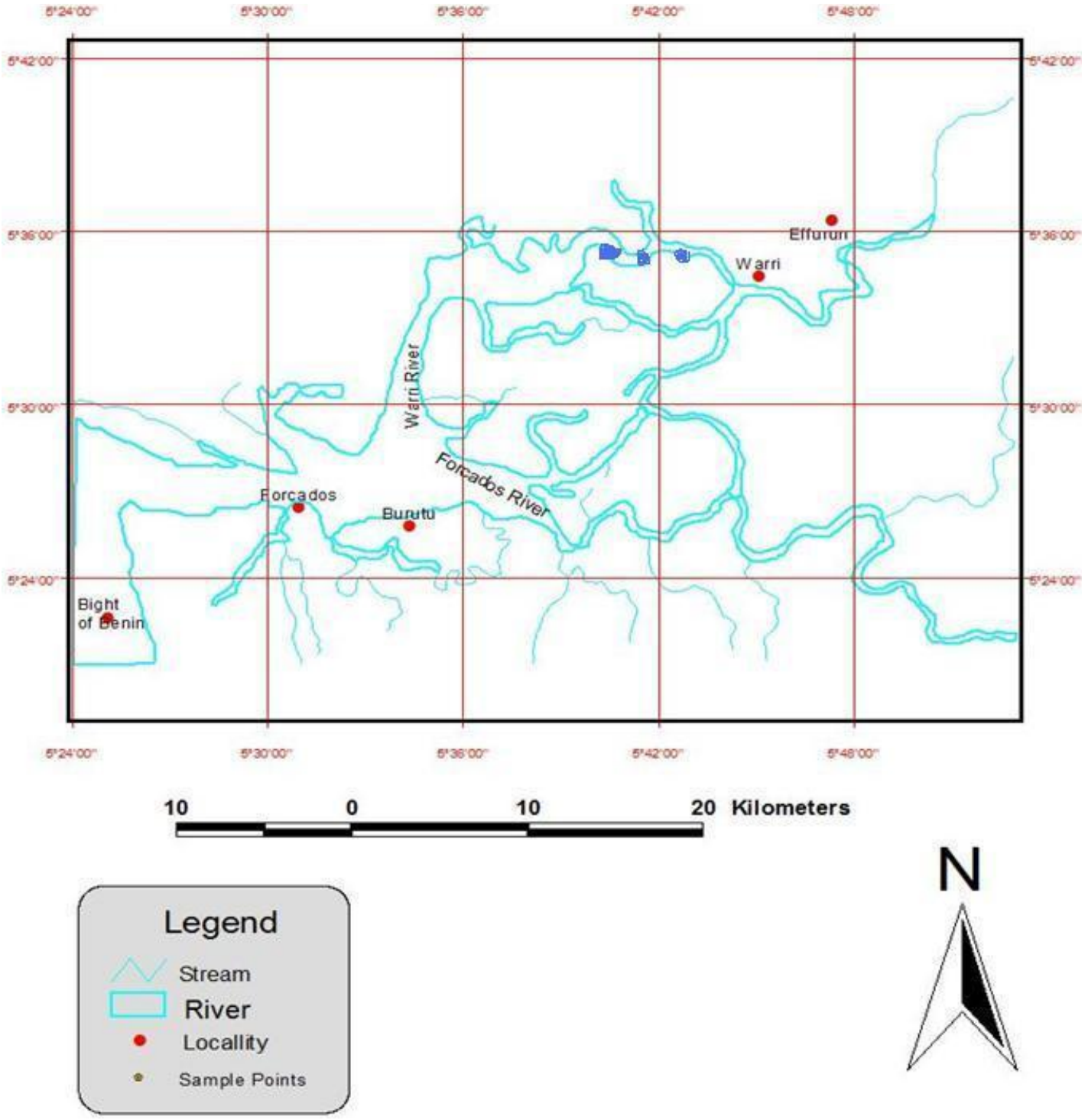

Figure 1. Sampling sites (painted in deep blue) at Ubeji in Warri River, Niger Delta.

Gel scoring and data analysis

Identified DNA bands were scored as 1 or 0 for their presence and absence respectively in each sample. Unambiguous and reproducible bands were used in analysis of the gels, hence, primers that produced no amplifications and bands were not used in analysis. These data were used to calculate genetic distance using Jaccard coefficient similarity matrices. Phylip (Felsenstein, 2005), a tool assessed through ExPASy, a web based bioinformatics resource of the Swiss Institute of Bioinformatics, was used to

Table 1. The sequences, GC% and annealing temperature of the RAPD primers used.

Primer	Sequence 5´- 3´	Annealing Tm (°C/S)
1	GAAACGGGTG	40/120
2	AATCGGGCTG	40/120
3	GGGTAACGCC	40/120
4	CAGCACCCAC	40/120
5	GTGATCGCAG	40/120
6	CCGGGAATCG	40/120
7	AGTCAGCCAC	40/120

Table 2. The sequences, GC% and annealing temperature of the specific primers used.

Primers sequences 5´ - 3´ (forward)	Sequences 3´ - 5' (Reversed)	Annealing Tm (°C/S)
GTGTGAATGTGCTTGTGTATGC	GTGCGGGGCAGTTATTGATGG	59.1/120
TGCATTTTTGTAGTGATGC	GGGCTCCTGGAGATTGC	59.1/120

Table 3. Summary of the number and characteristics of amplification products obtained from a survey of seven primers of random sequence among 11 individuals of the cichlid family.

Primer	NBF1	NBF2	NBF3	NBF4	NBF5	NBF6	NBF7	NBF8	NBF9	NBF10	NBF11
RAPD 1	1	2	0	0	0	0	0	0	1	0	1
RAPD 2	0	0	0	0	0	0	0	0	1	1	0
RAPD 3	5	2	2	1	1	5	0	4	5	2	3
RAPD 4	5	7	0	0	0	0	0	0	1	5	4
RAPD 5	4	4	2	4	1	1	4	1	3	4	5
RAPD 6	4	4	3	3	0	5	2	0	0	0	3
RAPD 7	0	0	0	0	0	0	0	0	2	1	0

NBF1-11, Number of bands in fish 1-11.

construct dendrogram using the Unweighted Pair-Group Method with Average linkages (UPGMA) method, with visualizations provided by PhyloWidget tool (Jordan and Piel, 2008).

RESULTS AND DISCUSSION

RAPD fingerprints for 15 individual fish specimens (coded F1-11 for cichlids and F12-15 for catfishes) using seven different RAPD primers (Table 1) were obtained. No residual DNA or artifacts were observed. The seven RAPD primers used produced fragments with varying bands and primers were individual specific in most of the species examined (Tables 3 and 4). The total number of bands generated by the seven RAPD primers (RAPD1-7) respectively was: 5, 2, 30, 22, 33, 24, 3 for the cichlids and 11, 8, 28, 8, 27 and 14 for the catfish family. The size of fragment amplified by these primers as shown in Tables 5 and 6 varied between 100 to 800 bp.

Of the total number of bands generated among cichlids (119), only 24% were polymorphic. Of 109 bands generated among catfishes, only 22% were polymorphic. An average of four polymorphic bands per primer was recorded. All primers yielded at least one polymorphic band except primer 2 among the cichlids. Also, primers 1, 2, 4 and 7 produced no amplification in several samples (Table 3) and thus were excluded in final analysis. The absence of amplified bands in significant number (for example two or more) of primers makes interpretation of polymorphism in such primers difficult, as the absence does not connote genetic similarity or otherwise (Hatanaka and Galetti, 2003). This underlines the importance of primer selection in using RAPD to assess genetic diversity. Primers 3, 5 and 6 (Table 1) had very intense and reproducible bands in all samples. The three primers generated 87 bands with 23 bands (26%) polymorphic and 74% conserved among cichlids. Among

Table 4. Summary of the number and characteristics of amplification products obtained from a survey of seven primers of random sequence for *Clarias gariepinus* species.

Primer	NF12	NF13	NF14	NF15
RAPD 1	0	4	4	3
RAPD 2	2	4	2	0
RAPD 3	8	9	7	4
RAPD 4	0	4	4	0
RAPD 5	6	7	7	7
RAPD 6	2	4	3	5
RAPD 7	0	2	2	2

NBF (12-15) Number of bands in fish 12 to 15.

Table 5. Summary of the number of polymorphism and size range of amplification products obtained from a survey of seven primers of random sequence among three *Tilapia* species from the Warri River.

Primer number	Total number of bands	Number of polymorphic band pair	Size range of base pair
1	5	1	400- 120
2	2	0	400
3	30	8	800- 100
4	22	4	800- 120
5	33	8	800-120
6	24	7	800- 100
7	3	1	600- 500

Table 6. Summary of the number and size range of amplification products obtained from survey of seven primers of random sequence for *Clarias gariepinus species* obtained from Warri and Ibadan.

Primer number	Total number of bands	Number of Polymorphic band	Size range of base pair
1	11	3	800-120
2	15	4	700-300
3	28	4	600- 110
4	8	2	400- 300
5	27	4	550- < 100
6	14	4	600- 110
7	6	3	600- 500

the catfishes, the three intense primers produced 69 bands with 16 (23%) polymorphic. All controls using specific primers were amplified.

A large number of DNA bands, 228 in all, were generated by the RAPD method used, with 24% being polymorphic loci. In particular, primers 3, 5, 6 and 7 produced a high level of polymorphism with unique sequences which were useful in distinguishing the cichlid and catfish families. This may be useful to distinguish evolutionary relationships and related species in higher taxonomic level (Soufy et al., 2009). In addition, primers 2 and 7 proved to be population specific for *Hemichromis fasciatus* (Table 3) and may serve as genetic markers.

Dendrogram analyses

Dendrogram was constructed (Figure 2) after data generated from the RAPD primer band analysis. The UPGMA cluster analysis of the similarity matrix based on RAPD data separated the cichlids specimens into three clusters and the catfishes one (Figure 2). The first (C) formed by two *T. zilli*, one *T. guineensis* and one *S. galilaeus*; the second (D) by one *T. zilli*, one *T.*

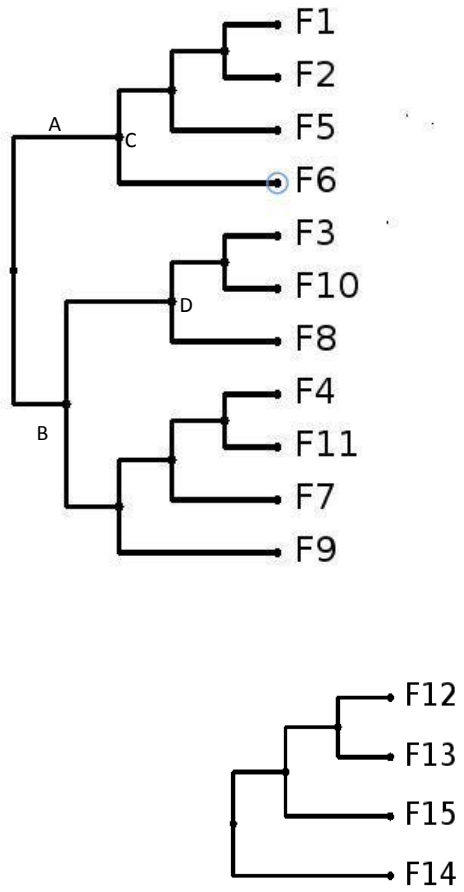

Figure 2. Dendrogram of finfish samples in Warri River at Ubeji plotted by Unweighted Pair-Group Method of Averages (UPGMA). Key for the leaves: F1-4 and 11, *Tilapia zilli*; F5, *Satherodon galileaus*; F6-8, *Tilapia guineensis*; F9-10, *Hemichromis fasciatus*; F12-15, *Clarias species.*

guineensis and one *H. fasciatus*; and the third (E) by two *T. zilli*, one *T. guineensis* and one *H. fasciatus*. The second and third clusters of the cichlids had smaller genetic distances and hence, a closer profile, forming clade B (Figure 2). Of the 11 leaves (specimens) of the cichlid dendrogram, seven were in clade B.

The Tilapia specimens showed genetic variation among themselves. Three of the *T. zilli* specimens - F3, F4 and F11 were of close distance to the *H. fasciatus* specimens F10 and F9. In particular, F3 shared a node and same branch length with F10, while F4 and F11 also clustered with F9. Also, all three specimens of *T. guineensis*- F6, F7 and F8 were in different clusters and were several nodes away from each other. The clustering of the *H. fasciatus* specimens followed a similar pattern. This rather wider variation than expected may be due to smaller number of primers that were eventually used for the dendrogram, as it reduces the primers available for complete analysis; however, the results may indicate a need for in-depth and large scale diversity study of cichlids at the study site.

In addition, F5, a *S. galilaeus* specimen had a profile close to that of F1 and F2, both *T. zilli* specimens (Figure 2). The dendrogram suggests that in each clade or cluster, there are always different taxa- no single species clustered together completely.

Several authors have demonstrated the effectiveness of the RAPD method in discriminating between species or subspecies in cases where close morphological characteristics and attributes have low resolving power (Ahmed et al., 2004; Saad et al., 2009). In this study, *T. zilli* and *T. guineensis* had different profiles from the RAPD primers, despite similar morphological features. In Figure 2, *T. guineensis* specimens F6, 7 and 8 formed a

separate branch at nodes C, D and E. Individual species possess qualities which make them appear similar morphologically but are different genetically as has been confirmed in this study.

Furthermore, there was also considerable variation in the RAPD profiles among the catfishes as shown in the dendrogram (Figure 2), with only two F12 and F13 showing very close genetic profiles by sharing a node. Overall, considerable genetic variation was observed within species (especially within *T.zilli, T. guineensis and C. gariepinus*), between species in the same genera (*T. zilli and T. guineensis)* and among cichlids and catfishes. Genetic variation observed in the study could be result of various factors such as differences in growth rate, fertility and phenotype of each genus and might also be due to individual ability to thrive in a highly contaminated environment. The Warri river site is close to a refinery and water bodies in the Niger Delta are believed to have experienced pollution (Akpan, 2013). However, the differences observed may be adduced to reasons such as hybridization among and between species in the wild, the peculiarities of the RAPD technique, or primer selection. Conclusively, we believe that the sensitivity of the RAPD technique with no prior knowledge of the genome played an important role in the detection of the observed differences in the fish samples.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Full Length Research Paper

In vitro multiplication of banana (Musa sp.) cv. Grand Naine

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A micropropagation method is described for banana (*Musa Spp*.) Cv. Grand Naine. Suckers were surface sterilized with $HgCl_2$ (0.1%) for 6 min which gave minimum contamination with maximum culture establishment. Of various treatment combinations, Murashige and Skoog (MS) medium + BAP 4.00 mg/l with IAA 2.00 mg/l resulted in maximum establishment of cultures in lesser time. MS medium + BAP 4.00 mg/l + IAA 2.00 mg/l gave maximum multiple shoots. Maximum rooting was obtained on MS medium (half strength) supplemented with IBA 1.00 mg/l and activated charcoal 200 mg/l.

Key words: Grand naine, micro propagation, murashige and skoog medium, *Musa* spp. and suckers.

INTRODUCTION

Banana is a perennial herbaceous monocot which belongs to *Musa* genus of the *Musaceae* family. It can be cultivated under sub-tropical conditions if the planting time is regulated in such a manner that bunches are initiated in summer, shot in autumn and mature in winter (Simmonds, 1996). It is believed to be one of the oldest fruits which have originated from Malaysia through a complex hybridization process (Novak, 1992). Cultivated banana is a triploid derived from two diploid species that is, *Musa acuminata (*Malaysia) and *Musa balbsiana* (India) (Georget et al., 2000). Banana plantlets produced through micro propagation method have been found to establish faster, healthier, stronger, shorter production cycle and higher yield than those produced through conventional methods (Ortiz and Vuylsteke, 1996) as millions of plants can be grown from a small or even a microscopic piece of plant tissue within a year (Mantell et al., 1985) and plants multiplication can be continued throughout the year irrespective of the season (Razdan, 1993). As regards yield performance in banana, tissue cultured plants have been reported to produce 39% higher yield than plants from sword suckers (Pradeep et al., 1992). The rapid proliferation obtained in tissue culture allows nurserymen to meet an unexpected demand for a particular cultivar; a million or more plants can be produced in a year from a single meristem tip. Another advantage of micropropagation is the elimination of pest and pathogen pressure during the production cycle, assuming that the initial stock plant is free of diseases (Faccioli and Marani, 1998).

In order to achieve the above objectives, several workers have developed tissue culture protocol and

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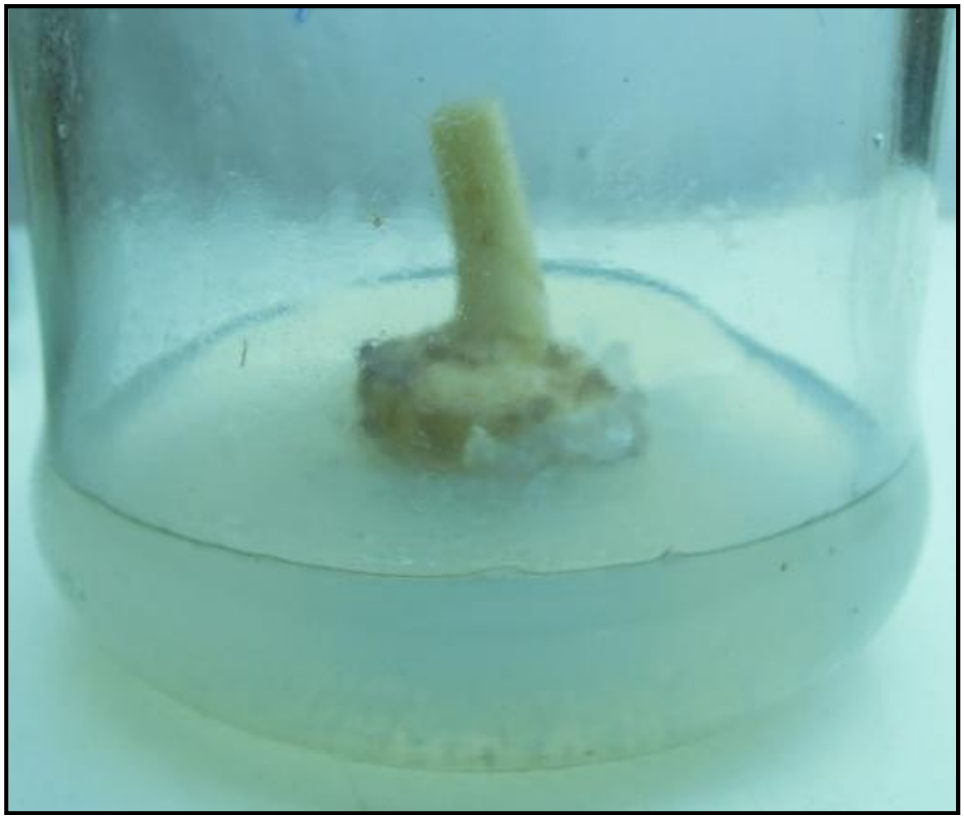

Figure 1. Culture initiation of banana explants cv. Grand Naine.

ascertained field performance of *in vitro* developed banana plantlets (Jalil et al., 2006; Resmi and Nair, 2007; Shirani et al., 2009). However, it is very important to standardize the technique under sub-Himalayan mountainous regions of Jammu because the performance of plant tissue is dependant on a number of factors which is intimately connected with the physiological state of the donor and the explants.

MATERIALS AND METHODS

The explant materials of Grand Naine cultivar of banana were obtained from plants grown at the Research Orchard of Division of Biotechnology, Punjab Agriculture University Ludhiana. The suckers were used as explant. The suckers were excised and surface sterilized by using different combinations and concentration of mercuric chloride, sodium hypochlorite and ethanol, individually or in combination which was followed by thorough rinsing with sterile distilled water.

Suckers were cultured on agar gelled MS medium with full strength salts supplemented with specific concentration of growth regulators (BAP, IAA and NAA) singly or in combination and 3% sucrose was used for culture establishment and shoot multiplication and MS half strength salts containing various concentration of auxins (IBA/NAA) were used for adventitious root formation. All the cultures were incubated in culture room at a temperature of 26 ± 2°C with relative humidity of 55 ± 5% and were exposed to 16 h photo period provided by 40 W cool white fluorescent tubes kept 50 cm above bench surface. Data recorded for different parameters were subjected to completely randomized design (CRD). Statistical analysis based on mean values per treatment was made using analysis of variance (ANOVA) technique of CRD. Number of treatments used varied according to the combinations of hormones used and number of replications was three. The software used for data analysis was O.P. State.

RESULTS

Surface sterilization

The study demonstrated the feasibility of propagating banana *in vitro* (Figure 1). The effect of different treatments of sterilants used either singly or in combination for the surface sterilization of explants is given in Table 1. Mercuric chloride (0.1%) for 6 min gave the best sterilization of explants, recording less (5%) contamination and the highest establishment (95%). Sterilization of explants with sodium hypochlorite alone was found to be ineffective resulting in very high contamination (55 to 100%) and also low survival percentage (0 to 15). On the other hand, considerable reduction in the rate of contamination (10%) with 85% survival of explants was obtained by treating the explants with sodium hypochlorite (5 %) for 10 min after rapid rinsing with ethanol (70%) for 30 s and mercuric chloride (0.1 %) for 6 min.

Table 1. Standardization of surface sterilization treatments for banana explants cv. Grand naine.

Sterilant	Duration	Contamination (%)	Death of culture (%)	Culture establishment (%)
Mercuric chloride (0.1%)	4 min	40(39.21)	30 (32.97)	30 (32.97)
Mercuric chloride (0.1%)	6 min	5 (12.64)	0 (0.00)	95 (77.53)
Mercuric chloride (0.1%)	8 min	50 (44.98)	25 (29.80)	25 (29.80)
Mercuric chloride (0.1%) + ethanol (70%)	4 min + 30 s	40 (39.15)	25 (29.88)	35 (36.14)
Mercuric chloride (0.1%) + ethanol (70%)	6 min + 30 s	15 (22.44)	10 (17.78)	75 (60.10)
Mercuric chloride (0.1%) + ethanol (70%)	8 min + 30 s	45 (42.04)	25 (29.82)	30 (32.88)
Sodium hypochlorite (5%)	5 min	100 (90.00)	0 (0.00)	0 (0.00)
Sodium hypochlorite (5%)	10 min	55 (47.87)	30 (32.88)	15 (22.65)
Sodium hypochlorite (5%) + Ethanol (70%) + Mercuric chloride (0.1%)	5 min + 30 s + 4 min	35 (36.14)	20 (26.35)	45 (42.05)
Sodium hypochlorite (5%) + Ethanol (70%) + Mercuric chloride (0.1%)	5 min + 30 s + 6 min	10 (17.78)	10 (18.30)	80 (63.61)
Sodium hypochlorite (5%) + Ethanol (70%) + Mercuric chloride (0.1%)	5 min + 30 s + 8 min	30 (32.97)	25 (29.86)	45 (42.04)
Sodium hypochlorite (5%) + Ethanol (70%) + Mercuric chloride (0.1%)	10 min + 30 s + 4 min	25 (29.78)	10 (18.06)	65 (53.83)
Sodium hypochlorite (5%) + Ethanol (70%) + Mercuric chloride (0.1%)	10 min + 30 s + 6 min	10 (17.23)	5 (12.37)	85 (67.24)
Sodium hypochlorite (5%) + Ethanol (70%) + Mercuric chloride (0.1%)	10 min + 30 s + 8 min	30 (32.88)	15 (22.65)	55 (47.95)
SE (m) ±		3.34	2.84	3.31
CD (0.05)		9.72	8.27	9.62

Values in bracket indicate that they are transformed values. Data recorded for different parameters were subjected to completely randomized design (CRD). Statistical analysis by (ANOVA) technique of CRD software used for data analysis is O.P. State.

Culture establishment

The result on number of days taken for establishment and per culture establishment obtained under each treatment are presented in Table 2. Figure 2 indicates that out of various treatments tried, the vegetative bud explants registered per cent establishment on MS medium supplemented with BAP (2.00, 4.00 and 6.00 mg/l) or BAP (2.00, 4.00 and 6.00 mg/l) in combination with IAA (2.00 mg/l). The time taken for culture establishment was less on the medium containing BAP in combination with IAA as compared to other treatments containing BAP alone or in combination with kinetin. The explants recorded least time (14.33 days) for culture establishment on MS medium supplemented with 4.00 mg/l BAP and 2.00 mg/l IAA. On the other hand, the treatment containing MS basal medium took significantly more time (25.00 days) for culture establishment.

Culture proliferation

In order to fix the optimum growth regulator concentration for shoot proliferation, the vegetative buds established on

Table 2. Effect of cytokinins and auxin on culture establishment of banana explants cv. Grand Naine.

Treatment	Time taken for culture establishment (days)	Culture establishment (%)
MS medium + BAP (2.00 mg/l)	18.00	85.33 (67.46)
MS medium + BAP (4.00 mg/l)	16.66	100.00 (90.00)
MS medium + BAP (6 .00 mg/l)	18.00	63.33 (52.73)
MS medium + BAP (2.00 mg/l) + IAA (2.00 mg/l)	15.00	87.66 (69.42)
MS medium + BAP (4.00 mg/l) + IAA (2.00 mg/l)	14.33	100.00 (90.00)
MS medium + BAP (6.00 mg/l) + IAA (2.00 mg/l)	16.33	76.00 (60.67)
MS medium + BAP (4.00 mg/l) + NAA (2.00 mg/l)	16.33	100.00 (90.00)
MS medium + BAP 4.00 mg/l + NAA 2.00 mg/l + IAA(2.00 mg/l)	14.66	100.00 (90.00)
MS Basal medium	25.00	41.00 (39.78)
SE (m) ±	0.14	0.06
CD (0.05)	0.44	0.20

Values in bracket indicate that they are transformed values. Data recorded for different parameters were subjected to completely randomized design (CRD). Statistical analysis by (ANOVA) technique of CRD software used for data analysis is O.P. State.

Figure 2. Culture establishment of banana explants cv. Grand Naine.

MS medium supplemented with BAP (4.00 mg/l) and IAA (2.00 mg/l) were re-cultured on the medium containing different concentration of growth regulators and the results on the frequency of explant showing multiple shoots, number of shoots per explant as well as length of longest shoot are presented in Table 3 and Figure 3. The frequency of the cultures showing multiple shoots ranged from 86.50 to 99% in treatment containing BAP (2.00, 4.00 and 6.00 mg/l) in combination with 2.00 mg/l IAA in which number of shoots ranged from 8.86 to 10.66 per culture. Further, the treatment combination of 4.00 mg/l BAPand 2.00 mg/l IAA gave maximum number of shoots (10.66) followed by 2.00 mg/l BAP and 2.00 mg/l IAA and were significantly superior to all other treatments.

Table 3. Effect of cytokinins and auxins on shoot proliferation of banana explants cv. Grand naine.

Treatment	Culture showing multiple shoot (%)	Number of shoots per culture	Length of longest shoot (cm)	Number of leaves on longest shoot
MS medium + BAP (2.00 mg/l)	56.00 (48.43)	4.66	8.10	7.33
MS medium + BAP (4.00 mg/l)	67.50 (55.23)	5.33	10.00	8.66
MS medium + BAP (6.00 mg/l)	52.50 (46.42)	3.33	7.30	6.00
MS medium + BAP (2.00 mg/l) + IAA (2.00 mg/l)	90.50 (72.16)	9.33	16.40	12.00
MS medium + BAP (4.00 mg/l) + IAA (2.00 mg/l)	99.00 (85.36)	10.66	18.30	13.00
MS medium + BAP (6.00 mg/l) + IAA (2.00 mg/l)	86.00 (68.03)	8.86	15.00	10.33
MS medium + BAP 2.00 mg/l + NAA 1.00 mg/l + IAA 2.00 mg/l	46.50 (42.97)	3.00	6.20	5.33
MS medium + BAP 4.00 mg/l + NAA 2.00 mg/l + IAA (2.00 mg/l)	77.50 (61.72)	8.00	14.30	9.33
MS medium + BAP 6.00 mg/l + NAA 3.00 m g/l + IAA 2.00 mg/l	39.50 (38.89)	2.00	4.50	4.00
SE (m) ±	1.48	0.17	0.84	0.22
CD (0.05)	4.44	0.52	2.51	0.67

Values in bracket indicate that they are transformed values. Data recorded for different parameters were subjected to completely randomized design (CRD). Statistical analysis by (ANOVA) technique of CRD software used for data analysis is O.P. State.

Figure 3. Culture proliferation of banana explants cv. Grand Naine.

Table 4. Effect of auxins and activated charcoal on *in vitro* rooting characters of banana plantlets cv. Grand naine.

Treatment	Time taken (days)	Culture rooting (%)	Number of roots per culture	Length of longest root (cm)	Length of shoot (cm)	Number of leaves per shoot
MS (half strength) + IBA (0.50 mg/l)	10.66	63.33 (52.71)	3.66	4.70	4.20	3.66
MS (half strength) + IBA (1.00 mg/l)	7.00	82.00 (65.62)	6.00	7.00	6.70	5.33
MS (half strength) + NAA (0.50 mg/l)	13.66	52.66 (46.51)	1.66	2.20	2.30	1.66
MS (half strength) + NAA (1.0 mg/l)	12.00	60.66 (51.15)	2.66	3.50	4.10	3.00
MS (half strength) + IBA 0.50 mg/l + AC 200 mg/l	8.00	79.33 (62.95)	5.33	6.00	6.20	5.00
MS (half strength) + IBA 1.00 mg/l + AC 200 mg/l	6.33	98.66 (83.70)	6.66	7.80	7.40	6.33
MS (half strength) + NAA 0.50 mg/l + AC 200 mg/l	12.66	55.66 (48.23)	2.33	3.40	3.70	2.33
MS (half strength) + NAA 1.00 mg/l + AC 200 mg/l	13.33	54.66 (47.66)	2.00	3.10	3.20	2.00
MS (half strength) + IBA 0.50 mg/l + NAA 0.50 mg/l	10.00	64.33 (53.33)	4.00	5.20	4.80	3.66
MS (half strength) + IBA 0.50 mg/l + NAA 1.00 mg/l	12.33	60.33 (50.96)	2.33	3.40	3.80	2.66
MS (half strength) + IBA 1.00 mg/l + NAA 0.50 mg/l	6.66	95.33 (77.69)	6.33	7.20	7.20	6.00
MS (half strength) + IBA 1.00 mg/l + NAA 1.00 mg/l	7.00	82.33 (65.12)	6.00	7.00	6.80	5.66
MS (half strength) + IBA 0.50 mg/l + NAA 0.50 mg/l + AC 200 mg/l	9.00	71.66 (57.83)	4.33	5.20	5.40	4.33
MS (half strength) + IBA 0.50 mg/l + NAA 1.00 mg/l + AC 200 mg/l	9.66	68.33 (55.73)	4.00	5.40	5.00	4.00
MS (half strength) + IBA 1.00 mg/l + NAA 0.50 mg/l + AC 200 mg/l	7.66	82.66 (65.51)	5.66	6.30	6.30	5.33
MS (half strength) + IBA 1.00 mg/l + NAA 1.00 mg/l + AC 200 mg/l	8.33	75.66 (60.53)	4.66	5.70	5.80	4.66
MS basal medium (half strength)	14.33	50.66 (45.36)	1.00	2.10	2.50	1.33
SE (m) ±	0.25	1.61	0.17	0.14	0.13	0.12
CD (0.05)	0.51	4.64	0.48	0.40	0.38	0.49

Values in bracket indicate that they are transformed values. Data recorded for different parameters were subjected to completely randomized design (CRD). Statistical analysis by (ANOVA) technique of CRD software used for data analysis is O.P. State.

Maximum length of shoot (18.30 cm) was obtained on the medium containing BAP (4.00 mg/l) and IAA (2.00 mg/l). In case of number of leaves on longest shoot BAP (4.00 mg/l) and IAA (2.00 mg/l) obtained highest of 13.00 leaves.

In vitro rooting

The data pertaining to the response of different auxins (IBA and NAA) on *in vitro* rooting of banana buds are presented in Table 4 and Figure 4. Out of 17 treatments

Figure 4. *In vitro* rooting.

tried, only 3 treatments (MS half strength) with IBA (1.00 mg/l) and activated charcoal showed maximum rooting of cultures (98.66%) which was at par with MS (half strength) with IBA (1.00 mg/l) and NAA (0.50 mg/l) registering 95.33% rooting. However, the shootlet took least time (6.33 days) for root initiation on half strength MS medium supplemented with 1.00 mg/l IBA and 200 mg/l activated charcoal. The maximum number of roots per shootlet (6.66) and the maximum length of root (7.80 cm) was produced on the medium containing 1.00 mg/l IBA and 200 mg/l activated charcoal. Treatments MS (half strength) augmented with 1.00 mg/l IBA and activated charcoal supported the maximum length of shoot (7.40 cm), which was significantly superior to the rest of the treatments tested. Considering the leaves per shoot, the maximum number of leaves (6.33) were produced on MS (half strength) supplemented with 1.00 mg/l IBA and activated charcoal. Addition of activated charcoal avoids callus formation at the base of the shoot before rooting.

DISCUSSION

Surface sterilization

Titov et al. (2006) studied that for *in vitro* propagation of banana cv. Kanthali, huge number of explants die due to microbial contamination; it studies that contamination free culture were established by $HgCl_2$ for 6 min followed by several washes in sterile water and obviated the need to develop extensive and complicated surface sterilization protocol, also Suneeta and Das (2008) studied that the investigation on the effect of surface sterilization agents showed variation in respect to their sterilizing property; out of sterilizing chemicals, calcium hypochlorate, hydrogen peroxide and bromine water did not show any good response and Jaisy and Ghai (2011) who worked on *in vitro* propagation of banana also found treatment of explants with $HgCl_2$ (0.1%) for 6 min most effective surface sterilization procedure registering maximum culture establishment with minimum contamination.

Culture establishment

The results obtained in the present investigation regarding culture establishment are supported by the findings of Muhammad et al. (2007) who worked on micropropagation of banana. Rahman et al. (2005) also found that in case of shoot multiplication of dessert banana, MS medium supplemented with 4.0 mg/l BAP, 2 mg/l NAA and 2.0 mg/l IAA to be the best. However, in the present investigation MS medium augmented with 4.00 mg/l BAP and 2.00 mg/l IAA gave best results registering per cent establishment and took least time for shoot initiation. The differences obtained in the requirement of phytoharmones as reported by different researchers and also in the present investigation may be attributed to the differences in the levels of endogenous phytoharmones, nutrients, metabolites and interaction between various factors.

According to Skoog and Miller (1957), quantitative interaction between diverse growth factor may have decisive role in organogenesis. Ammirato (1986) observed that the factors involved in the control of organogenesis in culture are more complex and plant hormones, organic and inorganic nutrient and osmotic concentration exert a performed influence on organogenesis.

Culture proliferation

Likewise, Muhammad et al. (2007) found highest response of shoot multiplication in 4 mg/l BAP and 2 mg/l IAA while Rahman et al. (2005) obtained maximum multiplication on treatment involving 4 mg/l BAP, 2 mg/l IAA and 2.0 mg/l NAA. However, in the present study, a higher level of BAP (4.00 mg/l) was found to be more suitable. The basic phenomenon involved in the explants establishment, induction of multiple shoots and subsequent plantlet production *in vitro* are reported to be due to action of plant hormones. Little is known about how hormones evoke the particular pattern of morpho-genesis (Thorpe, 1978). One hypothesis is that hormone treatment starts the cell on a specific development pathway; the alternative view is that hormone responsive cells are already determined and hormone evokes the expression of the combined state. Hence, the observed differences in the requirement of growth hormones for shoot proliferation of explant as reported by different researchers as well as in the present investigation could be attributed to the differential requirement of the growth hormones.

In vitro rooting

In vitro multiplication of banana is normally carried in the presence of high cytokinins levels, which inhibit root formation and elongation. Addition of 200 mg/l charcoal enhanced rooting and stopped callus formation. It was also obvious from the result that incorporation of activated charcoal reduced the time taken for root initiation and further increased the root and shoot length. Reports of Sharma et al. (1997); Gubbuk and Pekmezci (2006) and Roy et al. (2010) support the results as they obtained rooting only with 1.00 mg/l IBA and 200 mg/l activated charcoal.

Conflict of Interests

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

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Full Length Research Paper

Characterization of *Medicago* populations under cold acclimation by morphological traits and microsatellite (SSR) markers

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The study was carried on 16 accessions of annual *Medicago* species (*M. truncatula* Gaertn. *M. ciliaris* Krocker., *M. aculeata* Wild. and *M. polymorpha* L.). Seedlings of different accessions collected from sites of contrasting altitudes (10 to 1170 m) were subjected to different durations of low temperature regimes. Root to shoot ratios of acclimated and non acclimated plants were compared. Among the 16 accessions studied, 12 were used to assess the degree of genetic polymorphism by SSR microsatellites. Results show that accessions that originated from high altitude had a better root to shoot ratios and so had better ability to cold acclimation than accessions that originated from low altitude (lower ability to cold acclimation). Tests differentiation between species by fisher pair indicates that all species were different from each other. Results show the highest level of homozygosity for all species (> 80 %). Moreover, there were differences between populations of the same species of cold acclimation, which will encourage for a study of association between cold acclimation and molecular polymorphism.

Key words: Cold acclimation, root: shoot ratios, molecular polymorphism, annuals populations, *Medicago*

INTRODUCTION

The genus *Medicago* has emerged as an important experimental species and its species are important forage sources of the world. Species of *Medicago* are interesting because of their adaptability to different soil and climates, their good winter growth, self reseeding, their possible use in rotation with cereal and their low input requirements (Bullita et al., 1994). Annual species adapted to cold, can guarantee consistent production grazing replacing unproductive fallow. A sufficient level of cold tolerance might be needed to extend area of utilization of annual species of *Medicago* in environments with low winter temperatures. Environmental constraints

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Abbreviations: PL, Seedling length; **SL,** shoot length; **RL,** root length; **CA,** cold acclimated; **NA,** non-acclimated; **LG,** linkage group; **Tm,** melting temperature; **SSR,** single sequence repeat; **Rt/Tt,** root: shoot ratio.

are limiting factors for agricultural productivity and play an important role in the distribution of plant species across different types of environments (Singh et al., 2002;Dita et al., 2005; Zhang et al., 2008; Saibo et al., 2009). Cold is one of the most important constraints limiting and controlling seed germination, development of seedlings (post germination growth), the growth and development of the adult plant (Boyer, 1982; Mohapatra et al., 1989; Janda et al., 2007; Baruah et al., 2009; Kurt, 2010; Dias et al., 2010). Up to 15% of the world's agricultural production was lost to frost (Zou et al., 2007). During crop establishment, extreme temperatures can decrease plant emergence and lead to drastic losses in crop yield and quality (Kim and Tai, 2011; Avia et al., 2013).

Germination and growth of seedling were rarely targeted by breeders. Increased knowledge on mechanism of cold stress tolerance, at early stages, can lead to possible genetic improvements (Brunel et al., 2009; Dias et al., 2010). Many plants increase in freezing tolerance in response to low, non-freezing temperature, a process known as cold acclimation (Levitt, 1980; Guy, 1990; Thomashow, 1999; Oullet, 2007; Baruah et al., 2011; Pirzadah et al., 2014). During the progressive lowering of temperature (chilling temperatures), plants that tolerated frost (freezing temperature) were those able to adjust their metabolism and basic cell functioning biophysical constraints imposed by the transition to low positive temperatures (Houde et al., 2006). If the plant can adjust its cellular processes for a long time of exposure to low positive temperatures, it unlikely and highly become tolerant to freezing (Sakai and Larcher, 1987; Guy, 1990; Thomashow, 2010). Many morphological changes had been documented during the acquisition of cold tolerance in different species (Meyer and Badarudin, 2001; Castonguay et al., 2009; Castonguay et al., 2010; Baruah et al., 2011; Iraba et al., 2013). Colds tolerance can be evaluated by changes in morphological indices such root: shoot ratios (Hekneby et al., 2001; Thapa et al., 2008; Hund et al., 2008; Janska et al., 2010). Thapa et al. (2008) showed that its ability to adapt cold acclimates was evident by significant increase in freezing tolerance of two genotypes of *M. truncatula* with the exposure to specific cold acclimation regimes. Sutka and Galiba (2003) showed that plants acclimating at a temperature below 10°C can tolerate temperatures as low as -30°C. The organs of plants differed in their tolerance to cold. In general, cold-tolerant species (grasses, shrubs and grasses) had small size, low leaf area and root: shoot ratio significant. The root stem ratio was much higher in acclimated plants than in controls (Hekneby et al., 2001). Other authors, however, had stipulated that the roots were more sensitive than the collars (Mackersie and Leshem, 1994).

Phenotypic assessment can provide a direct and easy estimation of variability for cold stress adaptations. However, they were often affected by environment. Microsatellite markers, free from this constraint, were often used in combination with phenological traits to characterize populations and their adaptation to constraint environments (Dias et al., 2008; Badri et al., 2008; Touil et al., 2008; Lazerek et al., 2009; Li et al., 2009; Avia et al., 2013). Cui et al. (2013) reported that there was a positive association between morphological characters and SSR markers in relation to the origin of Japonica rice improved populations and their cold tolerance. Lazerek et al. (2009) investigated the genetic diversity of a collection of *M. truncatula* using a set of 18 microsatellites and found that seven had a correlation with altitude, rainfall and environmental salinity of origin of these populations. These authors also suggested that these markers loci linked to genes are involved in the adaptation to the altitude. Dias et al. (2008) highlighted some concordance between morphological characters and SSR markers in red clover (*Trifolium pratense* L.). Badri et al. (2008), studying the genetic variability in natural populations of *M. laciniata* Mill. (Fabaceae) found that the morphological diversity and molecular diversity were significantly associated with eco-geographical factors. Touil et al. (2008), studying the genetic diversity in cultivated populations of *M. sativa* L., highlighted that there was no correlation between SSR markers and the geographical origin of populations. However, these authors had emphasized that SSR markers are very informative and appropriate approaches in characterization and molecular polymorphism in populations of *Medicago*. In Algeria, annual populations of *Medicago* species were often used in rotation cereal-forage areas; growth of this species in these areas was seriously limited by the ability of each species to grow during cold winters. A sufficient level of cold tolerance might be needed and specific responses to these conditions are required to extend the area of utilization of this annual species. The aims of this project are therefore: to determine the ability and evaluate cold acclimation (CA) in range of annual accessions of *Medicago* representing broad geographic origins. CA was evaluated by measuring root to shoot ratios of treated plants at the different durations in comparison with the non-acclimated (control) and to estimate genetic diversity of this natural *Medicago* accessions using SSR markers and determine if there was a relationship between cold acclimation of populations and SSR markers used.

MATERIALS AND METHODS

Plant material, growth conditions and cold stress treatment

The study was carried on four annuals *Medicago* species (*M. truncatula* Gaertn. *M. ciliaris* Krocker., *M. aculeata* Wild. and *M. polymorpha* L.). A set of 16 accessions was tested for their ability of cold acclimation (Table 1). For growth conditions, ten seeds for each accession and repetition were germinated, after scarification, at room temperature in Petri dishes (85 mm) containing universal compost moistened with distilled water at the same conditions (16/8 (h) photoperiod (day/night), light intensity (6700 Lux)). A total of

4800 plants were treated. At three days growth stage, seedlings of each accession were divided into two lots; 150 in cold acclimation (CA) lot at 4°C for three durations 5, 8 and 11 days (T1, T2 and T3) and 150 in lot non-acclimated (NA) (control) kept at 23°C (T01, T02 and T03). The experimental design was a randomized plot with five replications. The total length of the seedlings (LP), the shoot (LT) and the root (LR) were measured at the end of each treatment on both the treated and control lots. LR and LT were used to calculate root: shoot ratios of cold acclimated and non-acclimated plants. Measuring root to shoot ratios at different durations in comparison with the control, were used to assess degree of cold acclimation at low temperature of the different accessions.

Statistical analysis

Statistical analyses were conducted using the Statistical Analysis System Statistica 6.1 version (Stat Soft, Inc. France). Data were analyzed by calculating mean and standard deviations values, for different settings and different treatments. Differences between the treatments means for each treat were performed using two-way ANOVA.

SSR analysis

Among 16 accessions studied, 12 populations were characterized using microsatellites markers (SSR). SSR analysis was carried out using 14 primer pairs originating from *M. truncatula*; selected based on their position on the genetic linkage map (Julier et al., 2003) and provided by the research unit URP3F/genetics INRA, Lusignan, France (Table 2).

DNA extraction and PCR

DNA was extracted from 200 mg samples of fresh young leaves from individual plants, in liquid nitrogen and 3 ml of 2% cetyltri-methylammonium bromide (CTAB) (BIOCHEM-CHEMOPHARMA. CANADA), according the technique of Doyle and Doyle (1990). PCR amplification was performed with 14 SSR microsatellites. The DNA from cultivars Magali, Mercedes, Jemalong and Gabes was extracted according to the method described by Cheung et al. (1993). PCR analysis was carried out at INRA, Lusignan France. PCR was performed for 10 µl total volume containing 1 × buffer, 02 mM dNTP, 0.2 µM SSR primers, 1.5 mM $MgCl_2$, 50 ng genomic DNA 0.3 units *Taq* polymerase (SIGMA-ALDRICH). Reactions were performed in a PTC-100 thermocycler (MJ Research, USA) programmed for an initial melting at 94°C for 4 min followed by 35 cycles at 94°C for 30 s, at melting temperature (Tm) for 1 min, a 72°C for 1 min, and then a final extension step at 72°C for 10 min. PCR products (2 µL/lane) were separated in 6.5% polyacrylamide gels in the LI-COR IR2 automated DNA sequence (LI-COR Inc.). Of the 14 SSR used, only nine of them were selected for which there was no missing data. For each primer pairs selected, alleles specifically detected in tolerant accessions were scored as (+). The different parameters were calculated using the GENETIX software (ver. 5.04) and GENEPOP (ver. 4.2).

RESULTS AND DISCUSSION

Growth evaluation among species of acclimated and non-acclimated seedlings

The results indicate that, among the four *Medicago* species, development for all traits measured at non-acclimated (control) and cold acclimated regimes varied both seedlings, shoots and roots lengths. ANOVA revealed highly significant differences ($p \leq 0.001$) among the populations studied between acclimated and non-acclimated lots (Table 4). These differences were more pronounced with treatment durations (Table 3). Under control generally, development of *M. ciliaris* was better than for the remaining three species (*M. truncatula, M. polymorpha* and *M. aculeata*). For example, for seedlings length, it varied on average from 9.57 to 13.83 cm (*M. ciliaris*) and from 5.88 cm to 6.89 cm (*M. polymorpha*) under control. Even under a low temperature regime, these species had a better development in comparison with the other three species. It varied from 6.20 to 3.32 cm (*M. ciliaris*) and from 3.51 to 4.48 cm (*M. polymorpha*). Compared to the control, radical length decreased significantly. This deceased was greater in *M. polymorpha* than others specified at all treatments. As stress persists over time, *M. aculeata* displays better root development.

Thus, the species *M. aculeata* and these populations were good candidates for improving cultivars tolerant to cold. Janska et al. (2010) had emphasized that species adapted by natural selection to cold environments have a good development root. Development of shoot was also affected by low temperature at the different durations. The decreases of this development were different from one species to another and from one treatment to another. *M. ciliaris* and *M. aculeata* had a rapid development (2.93 and 2.50 cm respectively) in comparison with M*. polymorpha* (1.80 cm) after five days under cold treatment. This trend seemed to be reversed after 11 days of cold treatment; *M. polymorpha* showed better development (2.55 cm) than *M. ciliaris* (1.73 cm).

Root to shoot ratio cold acclimation ability

In the literature, it was reported that there was a close relationship between tolerance to cold and frost and the ability of acclimation to low non-freezing temperatures. The degree of acclimatization was estimated by the root to stem ratio of treated plants to sub-zero temperature. Plants that displayed the most important ratios were considered cold-tolerant.

In this study, all accessions of the four *Medicago* species maintained at cold acclimated and non-acclimated (control) regimes varied for root to shoot ratio at the tree durations of treatment time (Figures 1, 2 and 3). In general, root to shoot ratio was higher in cold acclimated than in control. These results are in accordance with those that had been demonstrated by Thapa et al. (2008), which showed that root to shoot ratio was higher in cold acclimated than control plants in *Medicago truncatula.* For example, in *Medicago aculeata* genotypes, Ac 80 had a better ratio at the different

Table 1. Accessions analyzed for cold acclimation with their origin and ecological description. Cultivars, Mercedes, Gabes, Jemalong and Magali were used only as control in SSRs characterization by PCR.

Species	Population	Origin	Latitude	Longitude	Altitude (m)
M. aculeata Willd.	cv*. Ac 15678	Australia	-	-	-
	cv. Ac 15679	Australia	-	-	-
	cv. Ac 14821	Australia	-	-	-
	cv. Ac 80	Syria	-	-	-
M. ciliaris Krocker.	Cil 123	Algeria	36°46'02''N	8° 18' 9.57'' E	16
	Cil 124	Algeria	36°17'15'' N	7° 57' 14.77'' E	565
	Cil 125	Algeria	36°17'15''N	7° 57' 14.77'' E	565
	Cil 126	Algeria	36° 28' 0'' N	7° 26' 0'' E	290
M. polymorpha L.	Poly 57	Algeria	36°17'15''N	7° 57' 14.77'' E	565
	Poly 54	Algeria	36°17'15''N	7° 57' 14.77'' E	565
	Poly 136	Algeria	36°49'0'' N	5° 46' 0'' E	10
	Poly 213	Algeria	35°23'17''N	1° 19' 22'' E	1170
	Poly 42†	Algeria	36°54'15''N	7°45'07''E	200
M. truncatula Gaertn.	Tru 210	Algeria	34°6' 50'' N	2° 5' 50.14'' E	1150
	Tru 216	Algeria	34° 6' 50'' N	2° 5' 50.14'' E	1150
	Tru 62	Algeria	36° 28' 0'' N	7° 26' 0'' E	290
	Tru 26	Algeria	35° 23' 17'' N	1° 19' 22.16'' E	1170
M. sativa	cv. Magali	France	-	-	-
	cv. Mercedes	France	-	-	-
	cv. Gabes	France	-	-	-
	cv. Jemalong	France	-	-	-

*, Cultivars; †, accession having been only in the molecular characterization.

duration of cold acclimation (1.63, 2.31 and 1.40) than Ac 15679 (0.82, 1.10 and 1.30) respectively for T1, T2 and T3 (Figures 1, 2 and 3). Moreover, it seemed that populations from high altitudes were the best ratios under cold treatment compared to native lowland and T3 regime was most effective in distinguishing cold acclimation ability of accessions studied, especially for *M. truncatula* and *M. polymorpha* accessions. In *M. truncatula*, Tru 216, originating in high altitude (1150 m) (Table 1), showed the best ratio (1.20) in comparison with Tru 62 (0.69). The similar trend was observed in *M. polymorpha*, Poly 213, originating from 1170 m, which showed a ratio of 0.93 while Poly 57 originating from a low altitude (565 m) exhibited lower ratio (0.27).

Janska et al. (2010) showed that cold tolerant species - herbs, grasses and ground shrubs - had low leaf surface area and a high root: shoot ratio and cold-adapted plants tend to be slow growing. Thapa et al. (2008), in order to understanding cold acclimation of two contrasting genotypes of *M. truncatula* growing under low temperature regimes at different durations, observed that growing under low temperature regimes, comparatively to control conditions, resulted in a global growth reduction and root to shoot ratio was higher in cold acclimated than in control plants. Moreover, these authors related that tolerant genotypes had a higher root: shoot ratio and the behavior to the higher frost tolerance exhibited by tolerant ecotypes of *M. truncatula* after a low temperature period, highlight their greatest cold acclimation ability. In pea, Lejeune-Hénaut et al. (2010) reported that root: shoot ratio was higher under low temperature, particularly for the frost tolerant genotype Champagne. Bounejmate et al. (1994) revealed that there was a relationship between frost tolerance and winter temperature at site of collection for *M. aculeata*, with the most frost tolerant genotypes coming from high altitudes.

It appears that the populations from high altitudes areas presented superior ratio and thus had a great capacity for cold acclimation better than those of populations originating from lower geographical areas. The influence of the geographical origin of plants on their level of cold acclimation ability was often highlighted. Studying the growth of *M. truncatula* and *M. aculeata* genotypes collected from sites of contrasting altitudes and winter temperatures, Bounejmate et al. (1994) reported that there was a relationship between frost tolerance and winter temperature at site of collection, with the most frost tolerant genotypes coming from high altitudes and genotypes from high altitudes represent a promising source for breeding for first tolerance with greater variation in *M. aculeata* than *M. truncatula.* Avia et al. (2013) analyzed the genetic variability freezing associated to cold acclimation in range of accessions of *M. truncatula* representing broad geographic origins, and

Table 2. SSR primers used for annuals accessions of *Medicago* DNA amplification (INRA, Lusignan. France).

Marker	LG	Primer	*Tm* (°C)	Allele size in alfalfa (bp)
ATP456	3	L-GGGTTTTTGATCCAGATCTT R-AAGGTGGTCATACGAGCTCC	55	125
FMT-13	1	L-GATGAGAAAATGAAAAGAAC R-CAAAAACTCACTCTAACACAC	50	132
MTIC-79	5	L-AAAATCCAAAGCCCTATCACA R-AGCGTGAGATTTTTCCATCG	55	117
MTIC- 332	4	L-CCCTGGGTTTTTGATCCAG R-GGTCATACGAGCTCCTCCAT	55	124
MTIC- 338	3	L-TCCCCTTAAGCTTCACTCTTTTC R-CATTGGTGGACGAGGTCTCT	55	146
MTIC- 134	6	L-GCAGTTCGCTGAGGACTTG R-CAATTAGAGTCTACAGCAGCCAAAAACT	60	176
MTIC- 365	2	L-ATCGGCGTCTCAGATTGATT R-CGCCATATCCAAATCCAAAT	55	123
MTIC- 082	7	L-CACTTTCCACACTCAAACCA R-GAGAGGATTTCGGTGATGT	55	132
MTIC- 451	2	L-GGACAAAATTGGAAGAAAAA R-AATTACGTTTGTTTGGATGC	55	129
MTIC- 135	8	L-GCTGACTGGACGGATCTGAG R-CCAAAGCATAAGCATTCATTCA	55	123
MTIC- 343	6	L-TCCGATCTTGCGTCCTAACT R-CCATTGCGGTGGCTACTCT	55	134
MTIC- 131	3	L-AAGCTGTATTTCTGATACC R-CGGGTATTCCTCTTCTTCCTCCA	55	163
MTIC- 432	7	L-TGGAATTTGGGATATAGGAA R-GGCCATAAGAACTTCCACTT	55	163
B14B03	5	L-GCTTGTTCTTCTTCAAGCTC R-ACCTGACTTGTGTTTTATGC	55	151

Tm, melting temperature; LG, Linkage Groupe

Table 3. Average of the different traits measured under different treatments for all species.

Species	Treatment	PL(cm)	SL(cm)	RL(cm)
		Mean ± Std. Dev.	Mean ± Std. Dev.	Mean ± Std. Dev.
M. truncatula	T01	6.89 ± 2.69	3.52 ± 1.47	3.37 ± 1.68
	T1	4.84 ± 2.49	2.35 ± 1.33	2.48 ± 1.67
	T02	7.70 ± 7.70	4.08 ±2.02	3.61 ± 1.82
	T2	4.32 ± 2.58	2.19 ± 1.44	2.07 ± 1.47
	T03	8.15 ± 2.51	4.50 ± 1.73	3.64 ± 1.28
	T3	3.46 ± 3.13	1.79 ± 1.50	1.67 ± 1.85
M. ciliaris	T01	9.57 ± 3.87	4.62 ± 2.08	4.94 ± 2.41
	T1	6.20 ± 4.74	2.93 ± 2.45	3.26 ± 2.68
	T02	11.69 ± 3.45	5.92 ± 1.98	5.76 ± 2.15
	T2	4.92 ± 3.49	2.25 ± 1.54	2.67 ± 2.19
	T03	13.83 ± 2.57	7.20 ± 1.32	6.62 ± 2.02
	T3	3.32 ± 3.57	1.73 ± 1.83	1.58 ± 1.95
M. polymorpha	T01	5.88 ± 2.97	3.25 ± 1.88	2.62 ± 1.40
	T1	3.51 ± 2.16	1.80 ± 1.13	1.71 ± 1.45
	T02	6.52 ± 2.46	3.63 ± 1.54	2.88 ± 1.41
	T2	4.35 ± 2.30	2.30 ± 2.39	1.95 ± 1.23
	T03	6.89 ± 1.81	4.12 ± 1.26	4.12 ± 1.26
	T3	4.48 ± 2.40	2.55 ± 1.38	2.55 ± 1.38
M. aculeata	T01	7.42 ± 3.43	3.71 ± 1.92	3.71 ± 1.99
	T1	5.81 ± 8.04	2.58 ± 1.72	2.74 ± 2.10
	T02	8.85 ± 3.55	4.20 ± 1.88	4.64 ± 2.16
	T2	6.18 ± 3.43	2.69 ± 1.69	3.48 ± 2.38
	T03	9.90 ± 3.54	4.73 ± 1.83	5.16 ± 2.10
	T3	6.04 ± 3.78	2.60 ± 1.75	3.43 ± 2.26

PL, plant length; SL, shoot length; RL, root length; Std. Dev., standard deviation.

Table 4. Analysis of variance of different morphological parameters studied for cold acclimated and non-acclimated ecotypes.

Source of variation	ddl	Seedling length	Shoot length	Root length
		F	F	F
Ecotypes	15	12.95***	8.15***	18.96***
Treatment	5	70.14***	73.83***	56.62***
Ecotype* Treatment	75	1.99***	1.86***	2.38***
Error	4705			

*P ≤ 0.05; ** P ≤ 0.01; ***P ≤ 0.001; ns, non significant; F, coefficient of Fisher-Snedecor (test at level 5 %).

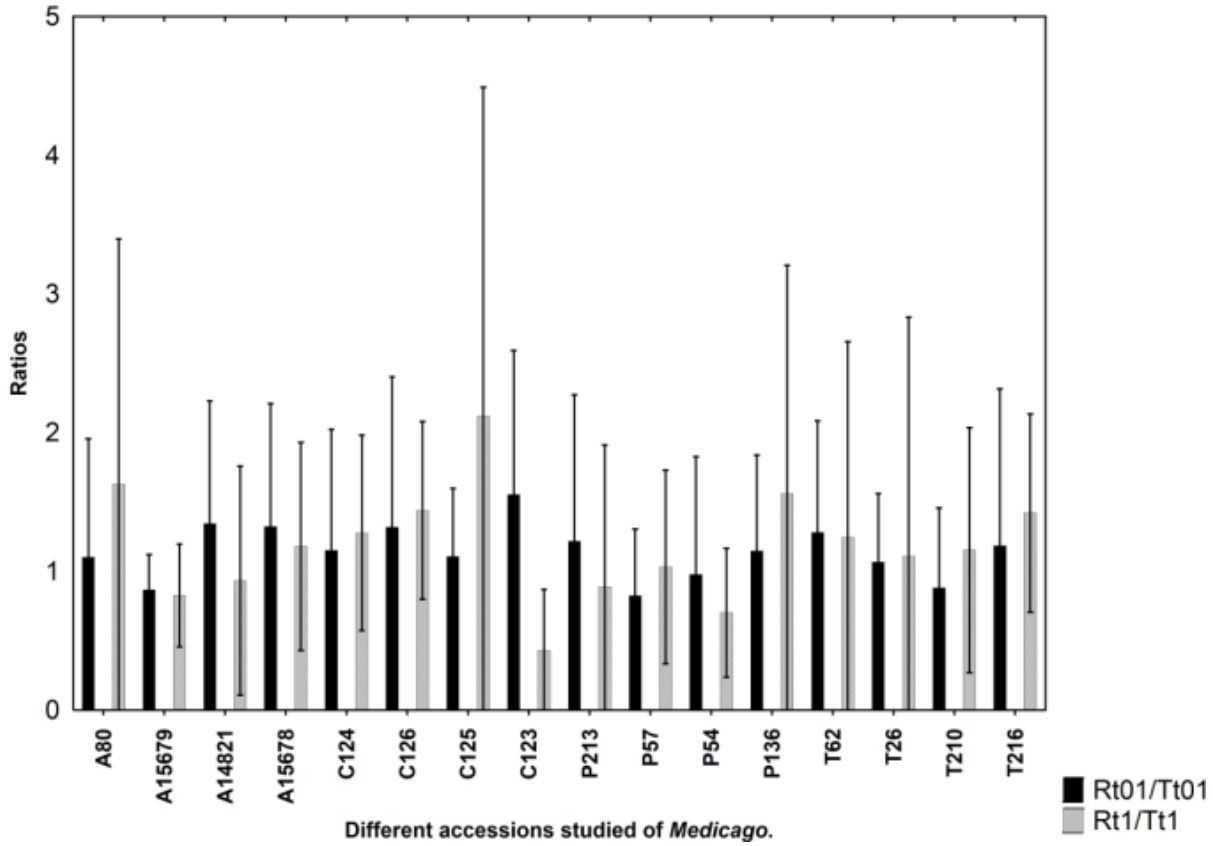

Figure 1. Ratios root to shoot after 5 days under 4 °C regime (Rt1/Tt1) and control (Rt01/Tt01), for different *Medicago* accessions studied.

revealed that accessions originating from higher latitudes were globally the most tolerant; which may reflect the adaptation tendency of accessions having evolved in geographical areas characterized by more frequent frost events.

Genetic diversity

Of the 14 SSR markers used, nine were selected (not missing data). The SSR loci used in our study were polymorphic. An example of SSR variation detected in populations studied is given in Figure 4. A total of 81 alleles were detected at the nine SSR loci for all species. The number of alleles detected per locus ranged from 6 (for ATPase456) to 13 (for Mtic432) (Table 5). Fisher tests differentiation between species pair showed that all species are different from each other. Results show the high level of homozygosity for all species (> 80%). The homozygosity percentage varied from 50 to 100% (Table

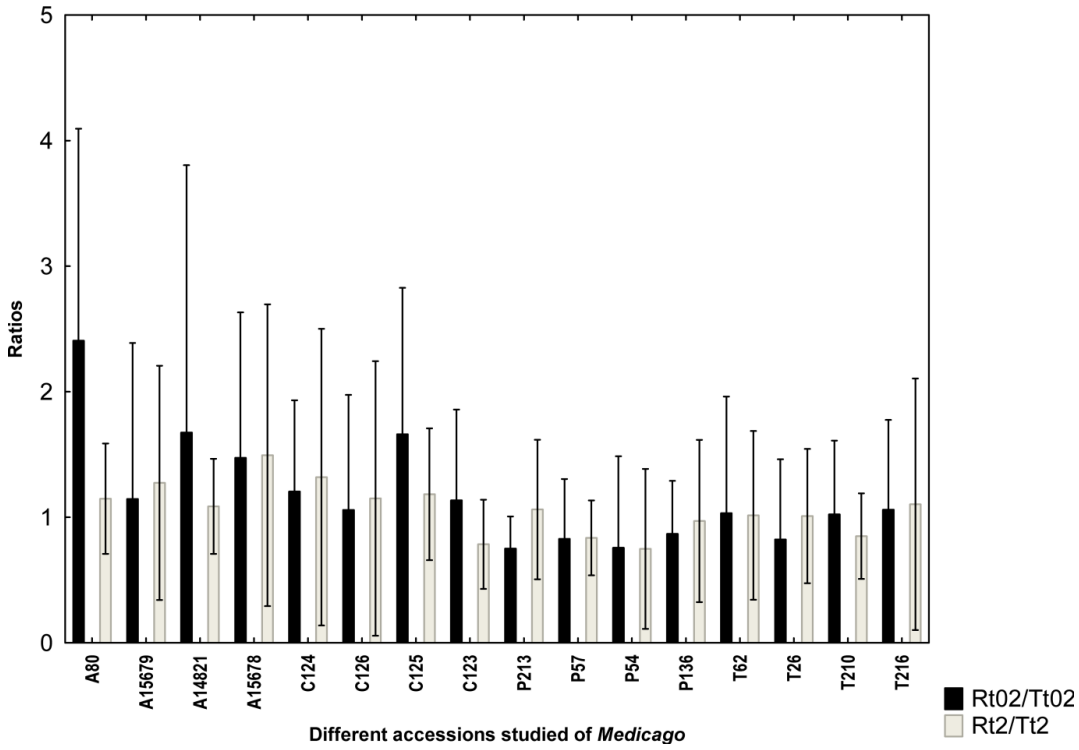

Figure 2. Ratios root to shoot after 8 days under 4 °C regime (Rt2/Tt2) and control (Rt02/Tt02), for different accessions studied of *Medicago*.

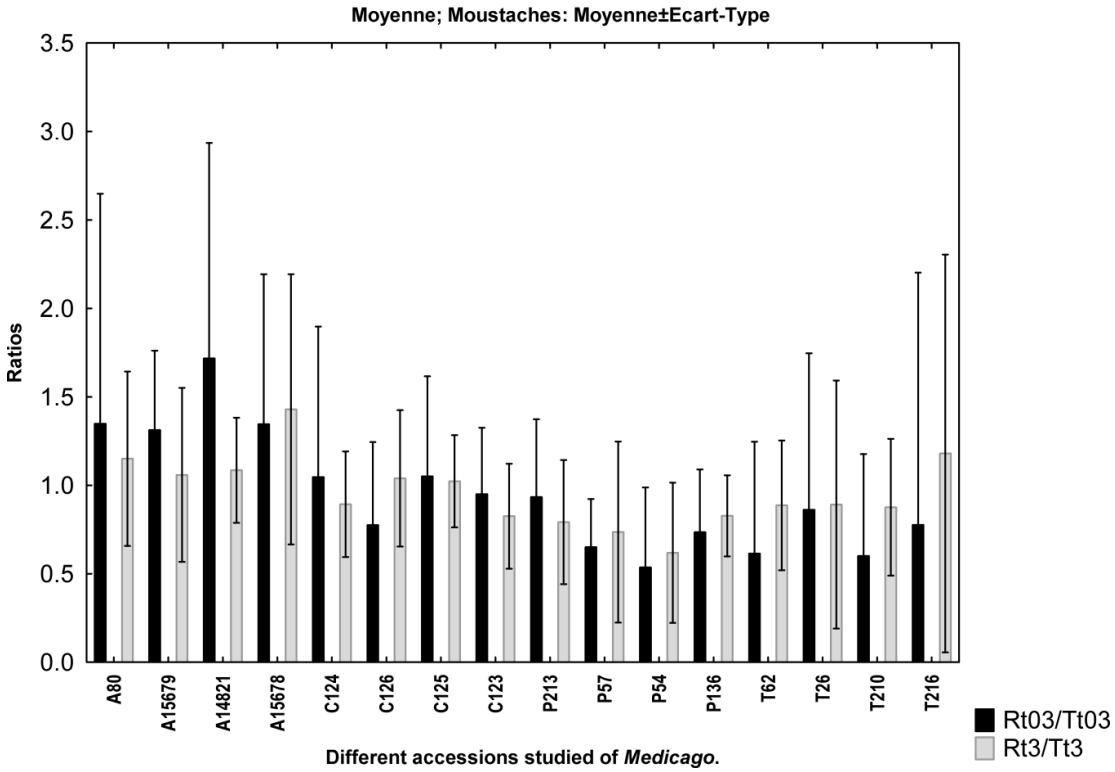

Figure 3. Ratios root to shoot after 11 days under 4 °C regime (Rt3/Tt3) and control (Rt03/Tt03), for different accessions studied of *Medicago*.

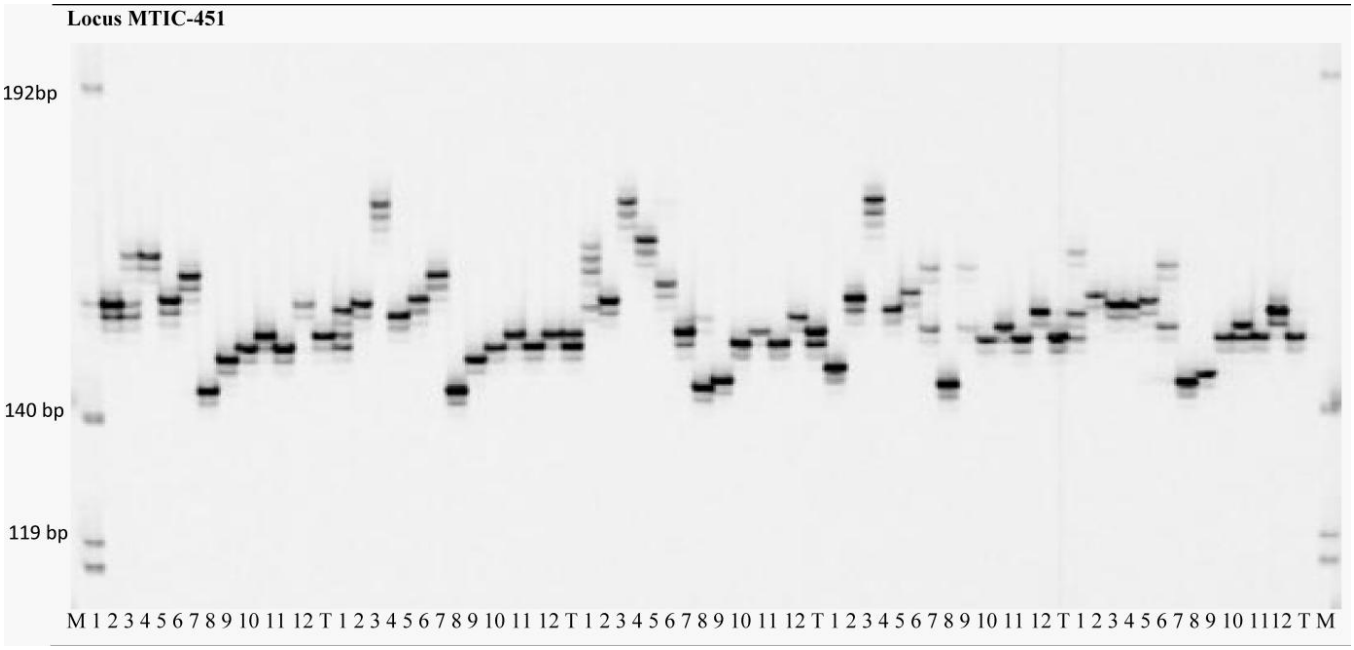

Figure 4. Example of SSR variation at MTIC-451 locus on twelve accessions of *Medicago* on five individuals. Black arrows indicate different alleles. M. marker leader (bp); 1-12: accessions, Cil 126, Cil 123, Cil 124, Tru 26, Ac 15679, Ac 80, Ac 15678, Poly57, Poly 213, Poly 42, Poly 136 and Poly 54 respectively and T : control, Mercedes, Gabes, Jemalong and Magali, respectively.

Table 5. Number of alleles detected among 09 markers loci selected and percentage of homozygosity.

Loci	*M. ciliaris*	*M. truncatula*	*M. aculeata*	*M. polymorpha*	All species
ATPase456	1(100%)*	1(100%)	5 (77%)	3 (50%)	6 (79%)
Mtic338	3 (85%)	1 (100%)	4 (92%)	5 (100%)	7(93%)
Mtic082	1(100%)	1 (100%)	2 (100%)	4 (67%)	7 (91%)
mtic451	5 (100%)	3 (100%)	5 (85%)	2 (75%)	12 (88%)
B14B03	1 (100%)	1 (100 %)	3 (100%)	5 (92%)	10 (98%)
mtic135	3 (92%)	2 (100%)	3 (100%)	2 (100%)	8 (98%)
mtic343	2 (100%)	2 (100%)	6 (92%)	6 (83%)	12 (93%)
mtic131	2 (92%)	2 (100%)	4 (100%)	4 (83%)	06 (93%)
Mtic432	1 (100%)	2 (80%)	2 (100%)	6 (83%)	13 (93%)
Total	19 (97%)	15 (98%)	34 (94%)	37 (81%)	81 (92%)

*The percentage of homozygosity is indicated in parenthesis

5). The overall level of polymorphism of *M. ciliaris* was lower than for *M. polymorpha* and *M. aculeata*. Moreover, it was possible to differentiate the four species with nine microsatellite markers and it was possible to differentiate between populations for *M. aculeata.* The population structure analysis showed that differences between populations of *M. aculeata* were similar to those between species and that the individual 12-1 is atypical; it seemed that it is an interspesific hybrid (Figure 5). In all alleles detected, allele 135 detected at markers loci Mtic131, alleles (187 and 190) detected at Mtic432 and alleles (133 to 154) detected at Mtic079 seemed to have a relationship with cold tolerance and the geographical origin of accessions.

Particularly, the alleles detected on the level of Mtic131 and Mtic-079 seemed to be specific to cold tolerant populations at both *M. aculeata* (Ac 15678, Ac 80 and Ac 15679) and *M. polymorpha* (Poly 57 and Poly 213) species (Table 6). Recently, Avia et al. (2013) found that these markers loci (Mtic131, Mtic432 and Mtic079) were linked to a QTL for cold tolerance in recombinant strains of *M. truncatula* from tolerant parents. They showed also in this recombinant, that strains of *M. truncatula* derived from crosses between two accessions acclimated to low

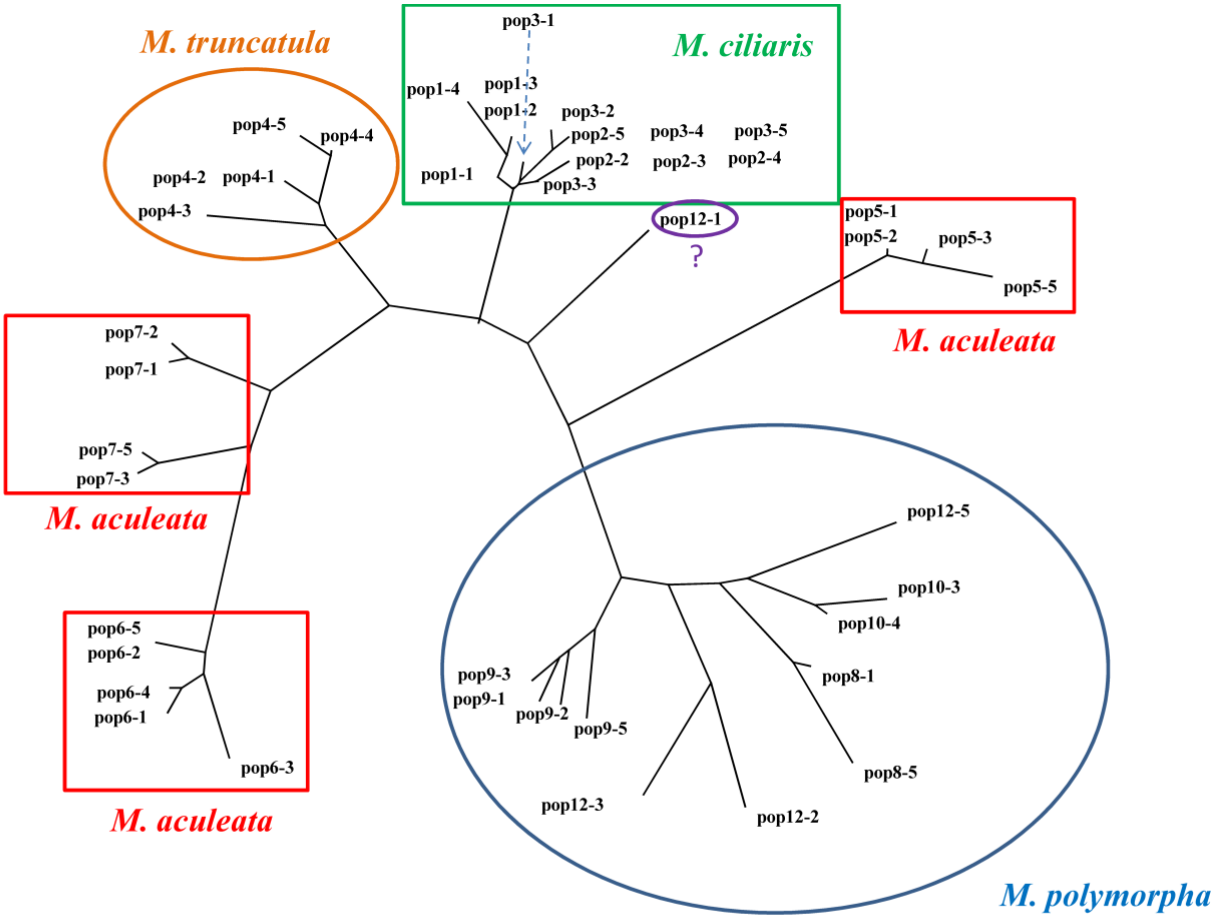

Figure 5. Nei standard Distance (1972), Neighbourg Joining, 1000 bootstraps, (no missing data). Pop1, Cil126; Pop 2, Cil 123; Pop 3, Cil 124; Pop 4, Tru 26; Pop 5, Ac 15679; Pop 6, Ac 80; Pop 7, Ac 15678; Pop 8, Poly 57; Pop 9, Poly 213; Pop 10, Poly 42; Pop 11, Poly 136 and Pop 12, Poly 54.

temperature and evaluation for cold tolerance by morphological characters showed

that the QTL associated with these markers (MTIC-131-432 MTIC, MTIC-079) had an additive effect and located at the liaison groups LG1, LG4 and LG6. These favorable effects of these additives alleles for cold tolerance were carried by both parents, suggesting that these ecotypes with a good ability to cold acclimation, effectively contributed to a good frost tolerance. These authors indicated that a relationship existed between the geographical origin of populations of *M. truncatula* and cold tolerance. Dias et al. (2008) highlighted some concordance between morphological characters and SSR markers in red clover (*Trifolium pratense* L.).

Bagavathiannan et al. (2010) investigating the genetic diversity of natural populations and cultivated alfalfa, find that there was correlation between phenotypic variables and SSR markers used. Badri et al. (2007) demonstrated in a study of genetic diversity among *Medicago ciliaris*, using morphological markers and locus SSR markers that the divergence of populations was due to local adaptation via genotype interactions / eco-geographical factors and these populations had a high plasticity to adapt too many different environments.

Conclusion

In conclusion, the investigation of cold acclimation ability at an early stage of development showed a significant variation between different populations for cold acclimation ability and cold tolerance. Moreover, this study demonstrated that accessions from high latitude areas had a high rate of root to shoot ratio. Ac 80, Ac and 15678 of *M. aculeata,* two populations of *M. polymorpha* Poly 136 and Poly 57, Cil 125 and Cil 126 of *Medicago ciliaris* and Tru 62 and Tru 216 of *M. truncatula* had been found that their degree of acclimation is more efficient for tree durations of treatment. This permitted to conclude clearly that Cold acclimation ability, at an early stage, was a good marker for cold stress tolerance. In total, 03 markers were identified that were associated with cold

Table 6. Alleles detected in tolerant accessions at marker loci Mtic432, Mtic079 and Mtic131, in parenthesis (size in bp).

Marker (allèles)	Accessions										
	Cil126	Cil123	Cil124	Ac15679 (T)	Ac80 (T)	Ac15678 (T)	Poly57 (T)	Poly 213 (T)	Poly 42	Poly136	Poly54 Tru 26
Mtic432											
(187)					+	+					
(190)				+		+					
Mtic079											
(133)				+	+		+				
(140)						+	+				
(154)				+	+						
Mtic131											
(135)				+		+	+	+			

(T), cold tolerant, **+**, allele detected only in the tolerant accessions

tolerance in two species, *M. polymorpha* and *M. aculeata*. Particularly, the microsatellite marker, Mtic-432, was identified only in *M. aculeata*. The fact that there was no structure between populations for *M. polymorpha* and *M. ciliaris* (to be confirmed with more individuals and markers) while there were differences between populations of the same species (*M. aculeata*) for cold acclimation was encouraging from the perspective of an association study between cold acclimation and molecular polymorphism.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Identification and characterization of acidity-tolerant and aluminum-resistant bacterium isolated from tea soil

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An acidity-tolerant, aluminum resistant bacterium was isolated from tea soils in Kagoshima Experimental Station (Japan). Based on the morphological, physiological and biochemical characteristics and 16S rDNA nucleotide sequence analysis, the bacterium was identified as *Bacillus* sp. An 3 (DQ234657) in *Bacillus cereus* group. The bacterium was able to grow on S-LB plates (pH 3.7) with 1.0 g/L Al and survived in LB broth even at 10 g/L Al (pH 2.0). While cultured, the growth of the bacterial strain in LB liquid medium containing increasing concentrations of Al (0, 100 and 200 ppm), was inhibited by the presence of Al, especially at concentration of 200 ppm. The pH of culture medium without Al increased steeply and reached pH 7.0 after 10 days, meanwhile it was almost constant in the other cases. The elimination of Al from culture medium by the bacterium was also affirmed and it was more conspicuous at 100 ppm Al. Due to their tolerance to high acidity, resistance to and removal of a substantial amount of Al, the bacterium might be applicable in restoring acidic soils, particularly acidified tea garden soils.

Key words: Tea garden soil, acidity-tolerant bacterium, aluminum-resistant bacterium.

INTRODUCTION

Green tea (*Camellia sinensis*) is a nitrophilic crop. As a farming practice, large amounts of nitrogenous fertilizers, especially ammonium sulfate fertilizer, have been applied to tea soils in order to increase the amino acid content of tea leaves and produce an attractively colored, tasty tea. When tea plants absorb a large amount of ammonium, sulfate accumulates in the soil. Also, ammonium applied to tea soil is rapidly converted to nitrate by acid-tolerant autotrophic nitrifiers (Hayatsu and Kosuge, 1993). Consequently, a considerable quantity of nitrate and sulfate has gradually accumulated in soil (Nioh et al., 1993), decreasing the pH to 4.0 or even lower and raising remarkably soluble aluminum (Al) levels (Wang et al., 2010). In these conditions, the activity of soil micro-organisms decreases and tea plants are considered to accumulate a high level of Al, thus posing a serious threat to the health of consumers (Fung and Wong, 2004). The utilization of soil microorganisms, which are indis-pensable participants in biogeochemical cycles, should be considered as a potential solution. Following this trend, it is necessary to know what happens to microorganisms living in the extreme environment of the tea garden

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soil or how microorganisms cope with the unfavorable conditions of this extreme environment. In other words, the study of the microbial ecology of the extremely acidic tea soil has promised to provide insights into environmental and applied aspects of indigenous microorganisms.

However, there have been few studies related to this aspect which are nitrification and autotrophic nitrifying bacteria in acid tea soils (Walker and Wickramasinghe, 1979; Hayatsu and Kosuge, 1993), acidity- tolerant and Al- resistant microorganisms (Konishi et al., 1994; Nioh et al., 1995; Kanazawa and Kunito, 1996; Kawai et al., 2000; Kanazawa et al., 2005; Takashi et al., 2012; Wang et al. 2013; Chao et al., 2013) and microbial activities in tea soil (Nioh et al., 1993; Hayatsu, 1993; Kamimura and Hayano, 2000; Koga et al., 2003; Han et al., 2007).

Therefore, this study purports to fill the gap of the microbial ecology of the extremely acidic tea soil. It aims at identifying and characterizing acidity-tolerant and Al-resistant bacteria isolated from acidic tea garden soil in Kagoshima (Japan).

MATERIALS AND METHODS

Soil samples

Samples of Kuroboku (high-humic Andosol), Akahoya (light-colored Andosol), Kuroniga (thick high-humic Andosol), Andesite-derived (weathered soil of Neogene layer in Tertiary Period) and Sedimentary rock-derived (weathered soil of Shimantogawa layer in Jurassic Period) soils were collected from tea gardens at a depth of 0 to 20 cm at the Kagoshima Tea Experimental Station. All the fresh soil samples were passed through a 2 mm mesh sieve (JIS standard), dried for 24 h, passed through a 0.5 mm mesh sieve (JIS standard) and kept in closed glass bottles for storage at 5°C.

Soil properties

Moisture content was based on the gravimetric loss of free water associated with heating to 105°C for 24 h. The pH (H_20) was measured with a PB-20 Sartorius Basic pH Meter and expressed as the ratio of air-dried soil to solution: 1:2.5. The amounts of total C and total N were determined using a N-C analyzer. The water-soluble Al in soils was extracted with pure water (1:20), followed by shaking for 2 h (Iwasaki et al., 1993), diluted with 1% nitric acid and then quantified by using Inductively coupled plasma-mass spectrometry (ICP-MS).

The total number of microorganisms was estimated by the dilution agar plate method on NA medium (beef extract 5 g, peptone 10 g, NaCl 5 g, agar 15 g, water up to 1000 ml, pH 4.0 and 7.0), for three to five days at 30°C. The coarse organic substances in the soil samples were dissociated by dispersion using a Waring Blender at 16,000 rpm for 3 min (Kanazawa et al., 1986). Cycloheximide was also spread on the surface of the plates to prevent the overgrowth of several rapidly growing fungi, which would restrict the growth of slower-growing molds.

Isolation of acidity-tolerant and Al-resistant bacteria

Bacteria tolerant to acidity and resistance to Al was isolated as follows. Autoclaved non-acidic field soil (100 g) was added to 1 L of distilled water and agitated for 30 min at 100 rpm. The mixture was filtered using a 0.50 µm filter. The filtrate was mixed with LB liquid medium (0.05% peptone, 0.025% yeast extract and 1% NaCl) to produce S-LB liquid medium. After this medium was autoclaved at 121°C for 15 min, Al sterilized using a 0.20 µm filter was added to a final concentration of 100 ppm, and the medium was adjusted to pH 3.7. The acidic tea soils (1 g) were added to 10 ml of this, and the culture was performed on a shaker at 150 rpm, 30°C for 7 days. The resulting bacterial strains were purified by incubation on S-LB agar plates (pH 7.0) (Konishi et al., 1994) (Method I). On the other hand, bacterial strains were directly isolated by the dilution agar plate method on S-LB (Method II) or LB agar plates (Method III), containing Al at a concentration of 100 ppm (pH 3.7). All the above-isolated bacterial strains were then transferred to S-LB agar plates containing Al concentrations of 200 to 1000 ppm (pH 3.7) and incubated at 30°C for seven days in order to screen for resistance to Al. Although small amount of yeast extract (0.2 g/L) and peptone (0.5 g/L) were included in the S-LB agar plates, their effects on the existence of inorganic monomeric Al were negligible (Kawai et al., 2000). The bacterium with higher ability to resist Al was selected for further analysis.

Identification

Identification was based on a morphological, physiological and biochemical characterization and phylogenetic analysis.

Morphological, physiological and biochemical characteristics

The tests were investigated on cultures grown at 30°C for 48 h. The bacterium was examined with an optical microscope for its cell form and size, Gram reaction, spore formation and motility. Colony form was observed on a medium plate. The catalase reaction, oxidase reaction, acid or gas production from glucose and oxidation or fermentation (O/F) of glucose were tested (Barrow and Feltham, 1993). Besides, physiological and biochemical characteristics were also determined using API 50 CHB kit (bioMerieux, Lyon, France) consisting of 49 carbohydrates of API 50 CH strip associated with the API 20 E strip.

Phylogenetic analysis

Colonies which developed on LB agar plates after 48 h at 30°C were harvested for analysis. InstaGene matrix was used for extraction and purification of genomic DNA, following its protocol. The nucleotide sequence (1500 to 1600 bp) of 16S rDNA of the isolate was amplified by PCR. The extracted genomic DNA acted as a template. Primers 9F and 1510R were added to Ready-To-Go PCR beads (Amersham Pharmacia Biotech, NJ, USA) which consist of deoxynucleotides, Taq DNA polymerase and PCR buffer to produce a complete PCR mixture. The nucleotide sequence of the amplified 16S rDNA was determined with an ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit. This kit was used with a GeneAmp PCR Systems 9600 thermal cycler and ABI Prism 3100 DNA Sequencer (Applied Biosystems, CA, USA). Eight kinds of sequence primers were used for the cycle sequencing. The sequences were screened for repeats, using an Auto Assembler 2.1 (Applied Biosystems, CA, USA) to rule out overlaps. The nucleotide sequence was analyzed by using MicroSeq Microbial Identification System Software V.1.4.1 (Applied Biosystems, CA, USA). MicroSeq Bacterial Full Gene Library v.0001 (Applied Biosystems, CA, USA) acted as a sequence database in similarity searches using the BLAST system (Saitou and Nei, 1987). Subsequently, a Neighbor-Joining molecular phylogenetic tree was constructed (Altschul et al., 1997). Then, in order to acquire more information, a similarity

search with the international nucleotide sequence database offered by U.S. National Center for Biotechnology Information (NCBI) using the BLAST was carried out. The nucleotide sequence data was submitted to GeneBank/DDBJ/EMBL for the accession number.

Bacterial tolerance to acidity and Al

To minimize the possible effect of soil eluate on the initial Al concentrations in the culture medium, the LB liquid medium was used in the following studies. $Al_2(SO_4)_3$ solution filtered with a sterilized filter (0.20 µm pore size) was added to the LB liquid medium autoclaved at 121°C for 15 min to final concentrations of 0.1 to 50 g/L^1, and the pH of the medium was adjusted to 2.0, 2.5, 3.0 and 3.5. The bacterial suspension (1 ml) was then inoculated into the medium and cultured by shaking at 150 rpm, 30°C for 7 days. After that, 50 µL of each culture was transferred onto LB plates in the absence of Al and heavy metals (pH 7.0), and continuously cultured at 30°C for three days. A positive test result under given culture conditions was affirmed via the development of colonies on these plates after incubating.

Culture conditions, bacterial growth and changes in medium pH

Cultures of 100 ml LB medium containing various concentrations of Al (pH 3.5) were inoculated with 1 ml of bacterial suspension and incubated by shaking at 150 rpm, 30°C for 10 days. During bacterial growth, the change of medium pH was measured with a PB-20 Sartorius Basic pH meter and the number of bacterial cells was counted by the dilution method on LB agar plates (pH 7.0).

Quantification of Al eliminated from culture medium

The spent culture supernatant was separated by centrifugation at 12,000 rpm for 10 min, then filtered with a sterilized filter (0.20 µm pore size), diluted with 1% nitric acid and subjected to ICP-MS analysis to determine the amount of Al remaining in the spent culture medium.

All the values represented the means of three independent experiments and were plotted along with their respective standard deviations. Differences of means were tested with Turkey-Kramer's method.

RESULTS

Soil properties

Several soil properties were determined (Table 1). The pH of tea soils varied in the range of 2.69-4.18. Soluble Al levels were significantly higher in the Kuroboku and Akahoya soil samples than in the other samples. The numbers of acidity tolerant microorganisms in the Kuroboku and Kuroniga samples probably increased due to the high acidity.

Isolation of acidity-tolerant and Al-resistant bacteria

Based on the differences in colony form, 41 bacterial strains which were able to tolerate pH 3.7 and 100 ppm Al was initially isolated. The result of the subsequent screening shows that two of these strains, namely Kb 1 and An 3, were able to grow on S-LB plates in the presence of 1000 ppm Al (Table 2). Therefore, strain An 3 derived from the Andesite-derived soil sample (pH 4.18) was selected for further research.

Identification

Morphological, physiological and biochemical characteristics

A photograph of strain An 3 is shown in Figure 1. The strain was a Gram-positive rod, 1.0 x 2.0-3.0 µm in cell size. This strain had motility and spore formation and was positive for both catalase and oxidase reactions. The characteristics of strain An 3 presented in Table 3 seemed to be in agreement with those of *Bacillus* genus. However, it was unlikely that this strain belonged to *B. mycoides* or *B. anthracis* which is included in the *B. cereus* group because they have no motility (Barrow and Feltham, 1993; Sneath et al., 1984). This is different from the above-mentioned suggestion based on the result of the nucleotide sequence analysis.

Besides, in physiological and chemical tests using the API 50 CHB kit, fermentation by strain An 3 of carbohydrate substrates such as ribose, glucose, fructose, arabinose, salicin, cellobiose, etc was detected, whereas that of others such as xylose, galactose, mannose, melibiose, raffinose, etc was not detected (Table 4). These characteristics of strain An 3 appeared to be similar to those of *B. cereus* and *B. thuringiensis* which were also contained in the *B. cereus* group. Although strain An 3 was considered to be closely related to *B. cereus* based on positivity for urease activity, however, their negativity of acetoin reaction (VP) was different.

In addition, in supplementary tests, strain An 3 was found to be positive in hemolysis, lecithinase and anaerobiosis, and negative in crystalline inclusion (Table 5). Based on these results, the possibility that strain An 3 belongs to *B. cereus* was greatest.

16S rDNA nucleotide sequence analysis

The nucleotide sequence of 16S rDNA of the bacterium was determined and presented in Figure 2. The result of the homology search with the MicroSeq Bacterial Full Gene Library using the BLAST system showed that the 16S rDNA base sequence of strain An 3 had more than 99% homology with that of *B. thuringiensis, B. cereus* and *B. mycoides* (Table 6). The result with the International Nucleotide Sequence Database using BLAST indicated 99.8% homology in 16S rDNA sequence with *B. cereus* H1439. Moreover, the first 20 hits in this

Table 1. Some properties of tea garden soil samples.

Soil sample	Depth (cm)	Moisture (%)	pH (H_2O)	Total C (g kg^{-1})	Total N (g kg^{-1})	C/N	Water soluble Al (mM kg^{-1})	Total number of microorganisms (10^6 g^{-1}) on NA medium	
								pH 4.0	pH 7.0
Kuroboku	0-20	28.32	2.69	9.57	0.582	16.4	9.45 ± 0.39	0.16	28.67
Kuroniga	0-20	26.91	3.11	17.36	0.880	19.7	6.77 ± 0.12	0.23	29.46
Akahoya	0-20	34.68	3.93	5.39	0.456	11.8	9.51 ± 0.35	0.12	31.38
Andesite-derived soil	0-20	38.48	4.18	2.70	0.270	10.0	2.77 ± 0.16	0.13	19.51
Sedimentary rock-derived soil	0-20	62.38	4.07	3.48	0.348	12.2	1.03 ± 0.40	0.09	3.90

homology list were related to *B. cereus* and *B. thuringiensis* (Table 7). Therefore, the possibility that strain An 3 belongs to *B. thuringiensis, B. cereus* or *B. mycoides* may be considered. However, since 16S rDNA nucleotide sequences of strain An 3 and these species do not entirely match, that the strain is closely related to another systematically different strain could not be absolutely excluded.

In general, five species, *B. thuringiensis*, *B. cereus*, *B. mycoides, Bacillus weihenstephanensis* and *B. anthracis* (Skerman et al., 1980) (anthrax, Bio Safety Level 3), are assigned to the *B. cereus* group with close relationships. In the Neighbor-joining phylogenetic tree constructed using MicroSeq (Figure 3), the cluster formed by strain An 3, *B. thuringiensis*, *B. cereus* and *B. mycoides* was considered to be the cluster of the *B. cereus* group (*B. weihenstephanensis* and *B. anthracis* were not registered in MicroSeq).

The 16S rDNA nucleotide sequence of strain An 3 has been deposited in the DDBJ/EMBL/GenBank database with the accession number DQ234657.

To sum up, the isolate may be identified as *Bacillus* sp. An 3 (with accession number DQ234657), part of the *B. cereus* group and related to *B. cereus, B. weihenstephanensis* or *B. thuringiensis*.

Tolerance to acidity and resistance to Al

Bacterial acidity tolerance and aluminum resistance in LB liquid medium were investigated. *Bacillus* sp. An 3 could survive in the presence of Al and low pH. As shown in Table 8, it could survive in the presence of Al up to 10 g/L at pH 2.0. This suggested that the strain was markedly tolerant to high acidity and resistant to Al.

Bacterial response to increasing concentrations of Al in culture medium

In this study, the growth of *Bacillus* sp. An 3 was influenced by the presence of Al in the culture medium, especially at an initial concentration of 200 ppm (Figure 5). In addition, during the growth, the pH of the culture medium without Al increased steeply and reached about 7.0 after 10 days, meanwhile it was almost constant at Al concentrations of 100 and 200 ppm (Figure 4). The result of the investigation on microbial elimination of Al from the culture medium showed that Al was removed by *Bacillus* sp. An 3 and it was more significantly conspicuous in the presence of 100 ppm Al than in the presence of 100 ppm Al (Figure 6).

DISCUSSION

Aluminum comprises 8.3% of the earth crust and is the most abundant metal and the third most abundant element after oxygen (45.5%) and silicon (25.7%). Aluminum appears in the Al^{3+} oxidation state and aluminum minerals are almost insoluble at neutral pH. As the pH drops below 5.5, however, Al-containing materials begin to dissolve. High levels of soluble Al in soils become toxic to plants and microorganisms (Mossor-Pietraszewska, 2001; Slattery et al., 2001). In order to deal with this, some microorganisms have developed mechanisms to tolerate high acidity and resistance to Al-stress conditions. In fact, a number of microorganisms tolerant to high acidity and resistant to Al from acidic soils have been isolated and identified (Konishi et al., 1994; Kanazawa and Kunito, 1996; Kawai et al., 2000; Nguyen et al., 2001; Kanazawa et al., 2005). However, it is remarkable that most of the microorganisms isolated were fungi and yeasts. This may be ascribed to the fact that fungi and

Table 2. Resistance to Al of acidity-tolerant bacteria.

Soil	Strain	Method of isolation	Al concentration (ppm)					
			100	200	300	400	500	1000
Kuroniga	Kn 1	(I)	+	-	-	-	-	-
	Kn 2	(II)	+	+	-	-	-	-
	Kn 3		+	+	-	-	-	-
	Kn 4		+	+	+	-	-	-
	Kn 6	(III)	+	+	+	-	-	-
	Kn 7		+	+	+	-	-	-
	Kn 8		+	+	+	+	-	-
	Kn 9		+	+	+	+	-	-
Kuroboku	Kn 10	(I)	+	+	-	-	-	-
	Kb 1		+	+	+	+	+	+
	Kb 2		+	+	+	+	+	-
	Kb 3	(II)	+	+	+	+	-	-
	Kb 4		+	+	-	-	-	-
	Kb 5		+	+	+	+	-	-
	Kb 6	(III)	+	+	+	+	+	-
	Kb 7		+	-	-	-	-	-
	Kb 8		+	+	+	+	+	-
Akahoya	Ah 1	(I)	+	+	-	-	-	-
	Ah 2		+	-	-	-	-	-
	Ah 3	(II)	+	+	-	-	-	-
	Ah 4	(III)	+	-	-	-	-	-
	Ah 5		+	+	+	-	-	-
	Ah 6		+	-	-	-	-	-
Andesite-derived soil	An 1	(I)	+	+	+	-	-	-
	An 2		+	+	+	+	+	-
	An 3		+	+	+	+	+	+
	An 4	(II)	+	+	+	-	-	-
	An 5		+	+	+	+	+	-
	An 6		+	+	+	+	-	-
	An 7	(III)	+	+	+	+	-	-
	An 8		+	+	+	+	-	-
	An 9		+	+	+	+	+	-
	An 10		+	+	+	+	-	-
	An 11		+	+	+	-	-	-
	An 12		+	+	+	+	+	-
Sedimentary rock-derived soil	Ts 1	(I)	+	+	+	+	-	-
	Ts 2	(II)	+	+	-	-	-	-
	Ts 3	(III)	+	+	+	+	-	-
	Ts 4		+	+	+	+	+	-
	Ts 5		+	+	+	+	-	-
Total	41		41	35	29	22	12	2

+ , Colonies; -, no colony grown at respective culture conditions.

yeasts are generally more tolerant to acidity than bacteria (Myrold and Nason, 1992; Pina and Cervantes, 1996). In addition to the acid- and Al- tolerant bacterial strain which was isolated and identified as *Flavobacterium* sp.

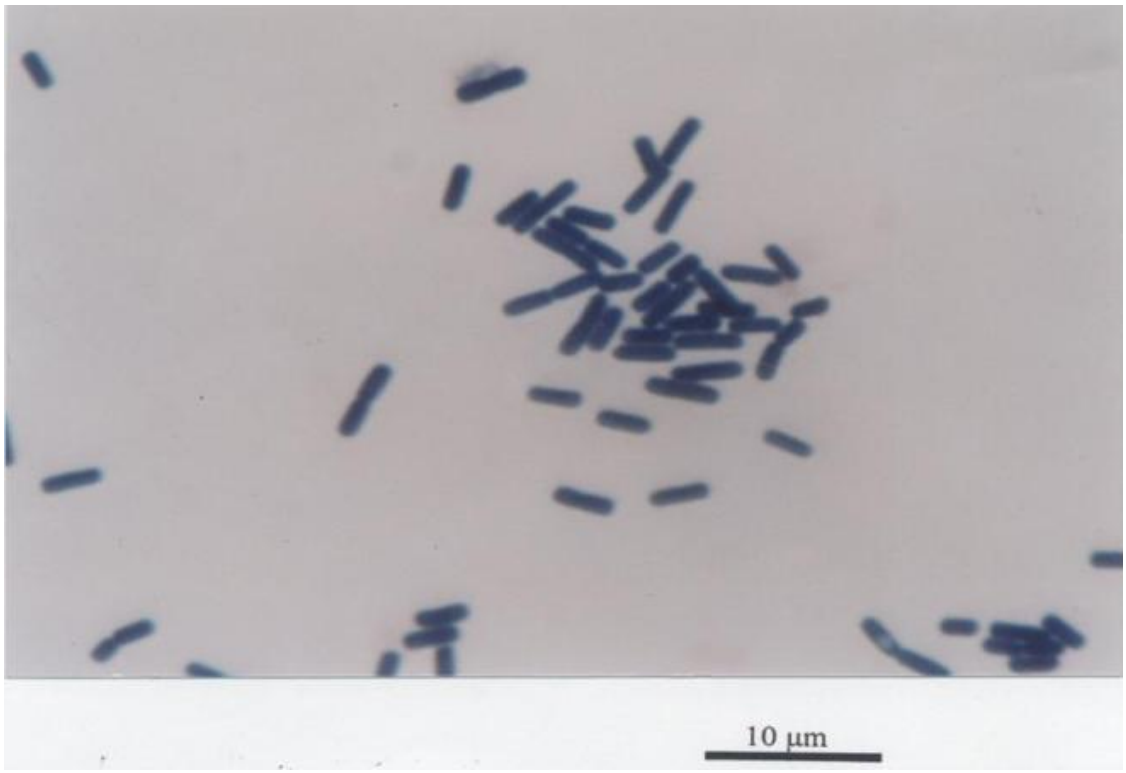

Figure 1. Microscopic characteristics of strain An 3.

Table 3. Morphological, physiological and biochemical tests.

Content		Strain An 3
Culture temperature (°C)		30
C Cell form and size (µm)		rod (1.0 x 2.0-3.0)
Gram reaction		+
Spore formation		+
Gliding motility		+
Colonial morphology		Culture medium: LB agar Culture time: 48 h Diameter: 1.0-2.0 mm Color: cream Form: ellipse Elevation: convex Margin: undulate Surface: smooth Opacity: opaque Texture: butter-like texture
Growth at (°C)	37	+
	45	-
Catalase production		+
Oxidase production		+
Acid/gas production from glucose		- / -
O/F test (Oxidation/Fermentation)		- / -

+, Positive; -, negative.

Table 4. Physiological and biochemical tests using API 50CHB Kit for the An 3 strain.

Tube	Test	Active ingredients	Reaction tested	Results
0		Control		−
1	GLY	GLYcerol	F/O	+
2	ERY	ERYthritol	F/O	−
3	DARA	D-ARAbinose	F/O	−
4	LARA	L-ARAbinose	F/O	−
5	RIB	D-RIBose	F/O	+
6	DXYL	D-XYLose	F/O	−
7	LXYL	L-XYLose	F/O	−
8	ADO	D-ADOnitol	F/O	−
9	MDX	Methyl-ßD-Xylopyranoside	F/O	−
10	GAL	D-GALactose	F/O	−
11	GLU	D-GLUcose	F/O	+
12	FRU	D-FRUctose	F/O	+
13	MNE	D-MaNnosE	F/O	−
14	SBE	L-SorBosE	F/O	−
15	RHA	L-RHAmnose	F/O	−
16	DUL	DULcitol	F/O	−
17	INO	INOsitol	F/O	−
18	MAN	D-MANnitol	F/O	−
19	SOR	D-SORbitol	F/O	−
20	MDM	Methyl-αD-Mannopyranoside	F/O	−
21	MDG	Methyl-αD-Glucopyranoside	F/O	−
22	NAG	N-AcetylGlucosamine	F/O	−
23	AMY	AMYgdalin	F/O	+
24	ARB	ARButin	F/O	−
25	ESC	ESCulin ferric citrate	F/O	+
26	SAL	SALicin	F/O	+
27	CEL	D-CELlobiose	F/O	+
28	MAL	D-MALtose	F/O	+
29	LAC	D-LACtose (bovine origin)	F/O	−
30	MEL	D-MELibiose	F/O	−
31	SAC	D-SACcharose (sucrose)	F/O	−
32	TRE	D-TREhalose	F/O	+
33	INU	INUlin	F/O	−

Table 5. Supplementary tests for bacterial identification.

Content	Strain An 3
Hemolysis test	+
Lecithinase activity	+
Crystalline inclusion	−
Anaerobiosis	+

(Konishi et al., 1994), in the present study, *Bacillus* sp. An 3 was able to survive in LB liquid medium containing a concentration of 10 g L^{-1} Al at pH 2.0. However, because of the various culture media, incubation conditions and assessment methods employed, it is difficult to make comparisons of Al resistance among bacteria from different studies.

It was reported that the bacterial adaptation to changes of medium pH may refer to the synthesis of an array of new proteins as part of what has been called their acidic tolerance response (Lansing et al., 2001). Furthermore, it was also proposed that either a high internal buffering capacity or reduced membrane permeability might play a role in pH homeostasis (Ian, 1985).

When pH decreases to 5.0 or lower, Al becomes soluble and toxic to microorganisms. The toxic effect of Al may be due to the substitution of essential metal ions at critical sites in the cell (Ganrot, 1986). However, the molecular mechanism of the toxicity has not been clarified. Here, the growth of *Bacillus* sp. An 3 was

1	gagtttgatc	ctggctcagg	atgaacgctg	gcggcgtgcc	taatacatgc
51	aagtcgagcg	aatggattra	gagcttgctc	tyawgaagtt	agcggcggac
101	gggtgagtaa	cacgtgggta	acctgcccat	aagactggga	taactccggg
151	aaaccggggc	taataccgga	taayattttg	aactgcatgg	ttcgaaattg
201	aaaggcggct	tcggctgtca	cttatggatg	gacccgcgtc	gcattagcta
251	gttggtgagg	taacggctca	ccaaggcaac	gatgcatagc	cgacctgaga
301	gggtgatcgg	ccacactggg	actgagacac	ggcccagact	cctacgggag
351	gcagcagtag	ggaatcttcc	gcaatggacg	aaagtctgac	ggagcaacgc
401	cgcgtgagtg	atgaaggctt	tcgggtcgta	aaactctgtt	gttagggaag
451	aacaagtgct	agttgaataa	gctggcacct	tgacggtacc	taaccagaaa
501	gccacggcta	actacgtgcc	agcagccgcg	gtaatacgta	ggtggcaagc
551	gttatccgga	attattgggc	gtaaagcgcg	cgcaggtggt	ttcttaagtc
601	tgatgtgaaa	gcccacggct	caaccgtgga	gggtcattgg	aaactgggag
651	acttgagtgc	agaagaggaa	agtggaattc	catgtgtagc	ggtgaaatgc
701	gtagagatat	ggaggaacac	cagtggcgaa	ggcgactttc	tggtctgtaa
751	ctgacactga	ggcgcgaaag	cgtggggagc	aaacaggatt	agataccctg
801	gtagtccacg	ccgtaaacga	tgagtgctaa	gtgttagagg	gtttccgccc
851	tttagtgctg	aagttaacgc	attaagcact	ccgcctgggg	agtacggccg
901	caaggctgaa	actcaaagga	attgacgggg	gcccgcacaa	gcggtggagc
951	atgtggttta	attcgaagca	acgcgaagaa	ccttaccagg	tcttgacatc
1001	gtctgaaaac	yctagagata	grgcttctcc	ttcgggagca	gagtgacagg
1051	tggtgcatgg	ttgtcgtcag	ctcgtgtcgt	gagatgttgg	gttaagtccc
1101	gcaacgagcg	caaccettga	tcttagttgc	catcattagg	ttgggcactc
1151	taaggtgact	gccggtgaca	aacoggagga	aggtggggat	gacgtcaaat
1201	catcatgccc	cttatgacct	gggctacaca	cgtgctacaa	tggacggtac
1251	aaagagctgc	aagaccgcga	ggtggagcta	atctcataaa	accgttctca
1301	gttcggattg	taggctgcaa	ctcgcctaca	tgaagctgga	atcgctagta
1351	atcgcggatc	agcatgccgc	ggtgaatacg	ttcccgggcc	ttgtacacac
1401	cgcccgtcac	accacgagag	tttgtaacac	ccgaagtcgg	tggggtaacc
1451	tttatggagc	cagccgccta	aggtgggaca	gatgattggg	gtgaagtcgt
1501	aacaaggtag				

Figure 2. The 16 S rDNA nucleotide sequence of An 3 strain.

Table 6. Homology search to MicroSeq Bacterial Full gene Library using BLAST.

Name of entry	Identity (%)
Bacillus thuringiensis	99.47
Bacillus cereus	99.40
Bacillus mycoides	99.34
Bacillus flexus	91.93
Bacillus cohnii	90.87
Bacillus oleronius	90.81
Bacillus atrophaeus	90.61
Bacillus megaterium	90.34
Bacillus horikoshii	90.34
Bacillus mojavensis	90.15

influenced by the presence of Al, especially at a concentration of 200 ppm. This may be ascribed to Al's toxic effect at high concentration to the bacterium. In order to deal with the toxicity, some microorganisms have developed mechanisms to tolerate metal-stress conditions. Mechanisms for metal detoxification include export, chelation and metabolism. The export and metabolism of Al have not been reported, while the tolerance of plants to Al is related to the secretion of organic acids, which chelate inorganic monomeric Al (Kochian, 1995). Additionally, acid- and Al- tolerant root nodule bacteria produce a larger amount of exopolysaccharides (EPS) than sensitive strains under stress (Appanna, 1988). It has been indicated that the production of EPS is a strategy to neutralize the toxic effects of Al (Appanna, 1989), since an EPS capable of chelating Al may substantially decrease the activity of toxic ions on the cell surface (Cunningham and Munns, 1984). Besides, it has been suggested that the acid- and Al- tolerant isolate, *Flavobacterium* sp. ST-3991, released certain substances, perhaps protein and chelators, during

Table 7. Homology search to international nucleotide sequence database using BLAST.

Name of entry	Name of strain	Accession no.	Identity
Bacillus cereus	G8639	AY138271	1507/ 1512= 99.7%
Bacillus cereus	H1439	AY138270	1509/ 1512= 99.8%
Bacillus cereus	G9667	AY138273	1506/1512= 99.6%
Bacillus cereus	2000031486	AY138272	1506/1512= 99.6%
Bacillus thuringiensis	2000031482	AY138290	1504/1512= 99.5%

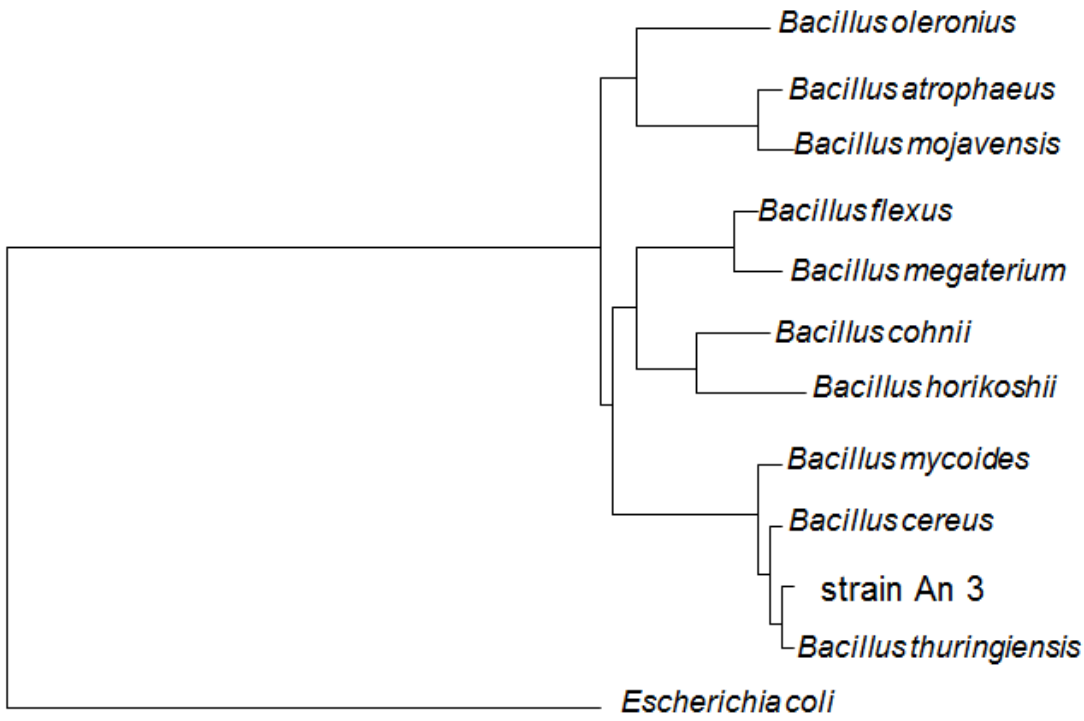

Figure 3. The Neighbor-joining molecular phylogenetic tree of strain An 3.

Table 8. Tolerance to acidity and Al of *Bacillus* sp. An 3.

pH	Al concentration (g L^{-1})						
	0	0.1	0.5	1.0	5.0	10.0	50.0
3.5	+	+	+	+	+	+	-
3.0	+	+	+	+	+	+	-
2.5	+	+	+	+	+	+	-
2.0	+	+	+	+	+	+	-

its growth, which might mask ionic Al and increase the pH of the medium. The masked Al appeared to form Al complexes because the culture medium became turbid and very viscous during the growth (Konishi et al., 1994). However, in the present study, during the growth of *Bacillus* sp. An 3, the pH of the medium without Al increased steeply and was neutral after 10 days, but that of the culture medium with Al was almost gradually decreased. This difference suggested the existence of a mechanism of responding to an increasing concentration of Al in the culture medium. However, elucidation of the precise mechanisms requires further study.

From acidic tea soils in Kagoshima in Japan, an acidity-tolerant and Al- resistant bacterium was isolated. The

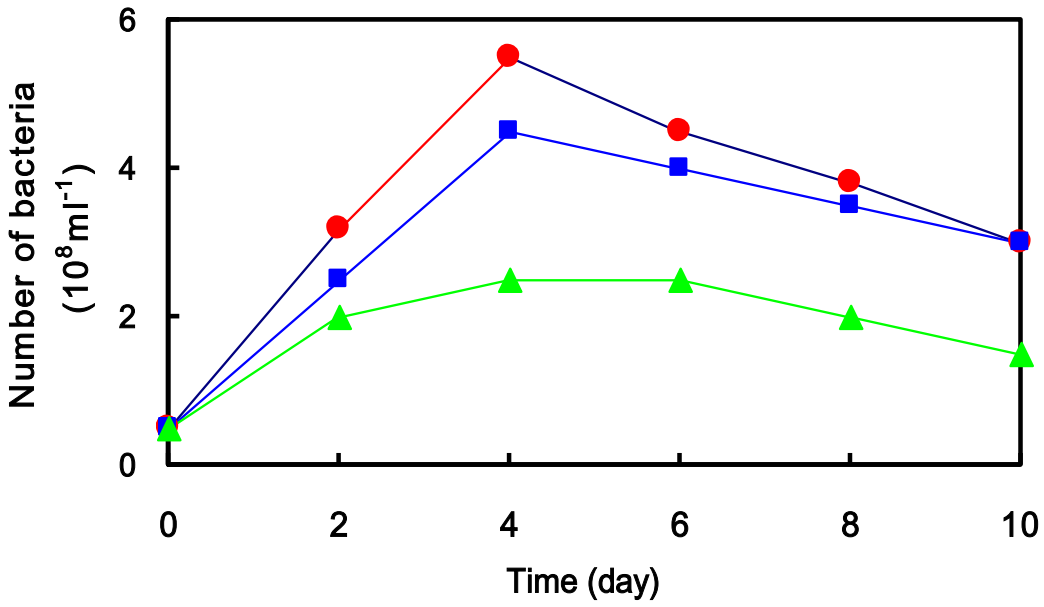

Figure 4. Growth of strain *Bacillus* sp. An 3 in LB liquid medium (pH 3.5) without Al (●), 100 ppm Al (■) and 200 ppm Al (▲).

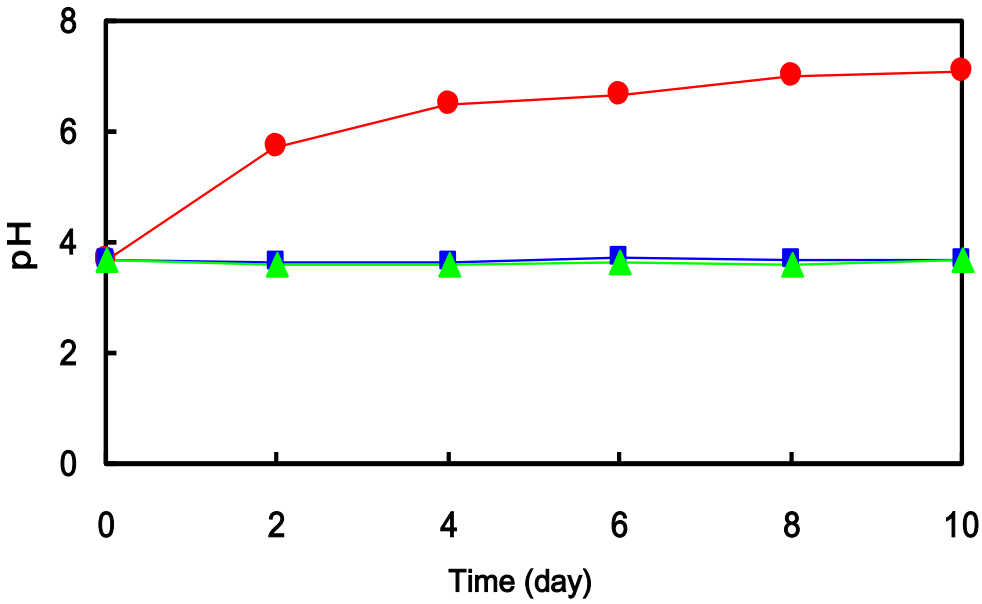

Figure 5. Changes of pH in response to increasing concentrations of Al in LB liquid medium (pH 3.5) without Al (●), 100 ppm Al (■) and 200 ppm Al (▲) of strain *Bacillus* sp. An 3.

isolate was identified as *Bacillus* sp. (with accession number DQ234657) and related to *B. cereus* and shown to tolerate high acidity, and to resist and eliminate a substantial amount of Al from the culture medium. These results not only have contributed to clarifying the characteristics of microbial ecology in acidified tea garden soil but also may facilitate studies on utilizing indigenous microorganisms to improve the current condition of tea garden soils.

Conflicts of Interest

The authors declare that they have no conflict of interest.

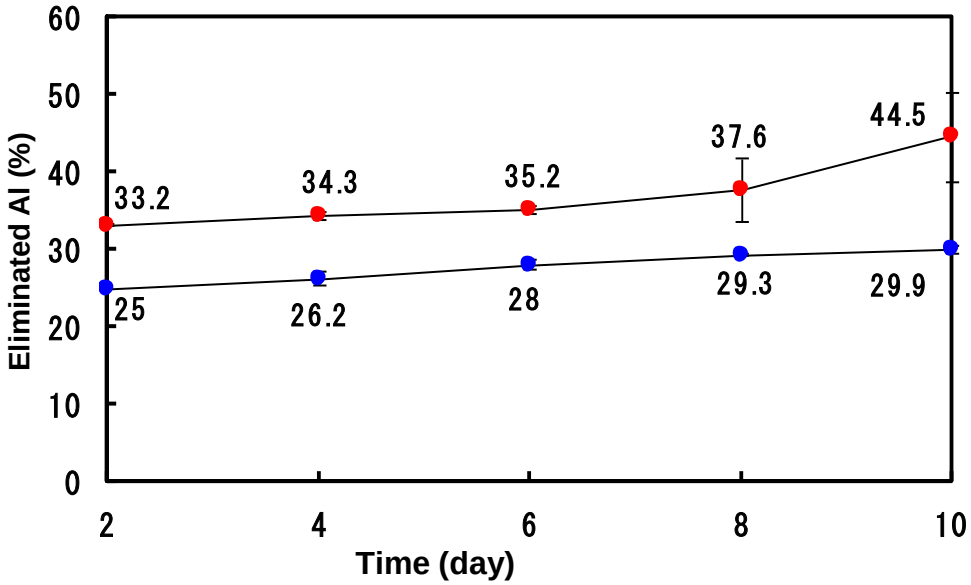

Figure 6. Elimination Al from LB medium (pH 3.5) containing 100 ppm Al (●) and 200 ppm Al (●) by strain *Bacillus* sp. An 3.

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Full Length Research Paper

Antifouling potential of seaweed, sponge and cashew nut oil extracts against biofilm bacteria and green mussel *Perna viridis* from Vellar estuary, Southeast coast of India

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Two species of common seaweeds and a single species of sponge were collected from Tuticorin coast and screened for antifouling activity. The seaweeds, *Sargassum wightii*, *Ulva lactuca*; sponge *Desmopsongiae* sp., and cashew nut oil extracts were tested *in vitro* against ten marine fouling bacteria isolated from test panels and the green mussel *Perna viridis*. The biofilm bacteria growth was inhibited by methanol extracts of the seaweeds *S. wightii*, *U. lactuca,* sponge *Desmospongae* sp., and the tropical cashew nut oil extracts. The bacterial growth was strongly inhibited by using extract concentrations as low as 30 µg mL^{-1} with *S. wightii*, *U. lactuca, Desmospongiae* sp., and cashew nut oil. The byssus thread formation of the mussel was completely inhibited by methanol extracts of *S. wightii, U. lactuca* and cashew nut oil extracts at concentrations of 100 µg ml^{-1}. These extracts showed strong antifouling activities on green mussel attachment with 100 µg ml^{-1} concentration. In this present study, there are exhibited preliminary evaluation of novel antifouling agents from marine macroalgae and tropical cashew nut oil.

Key words: *Perna viridis*, marine fouling bacteria, macroalgae, antifouling and tropical.

INTRODUCTION

All natural and man-made surfaces that are immersed in marine environments are potentially affected by the attachment of epibiotic and fouling organisms. The process of fouling begins as soon as a substratum is immersed with the adsorption of macromolecules, predominantly proteins, lipopolysaccharides and polysaccharides (Terlizzi et al., 2001). Subsequent microfouling and macrofouling by microbial slimes, algae, and invertebrates can result in major economic lost through, for example, corrosion (Little et al., 1999) and a reduction in the fuel efficiency of ships underway due to increased drag. Biofouling control is a worldwide problem in marine systems, which costs the U.S. Navy, for example, an estimated $1 billion per annum (Callow and Callow,

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2002). One of the most promising alternative techniques to tributyltin is the development of naturally occurring antifouling compounds from marine organisms. While some seaweed is heavily fouled, other species in the same habitat are rarely epiphytised, indicating the presence of antifouling mechanisms.

Marine natural products or extracts with antifouling activities have been isolated from a wide number of seaweeds. Compounds which have antifouling activity include, tannins extracted from *Sargassum natans* (Sieburth and Conover, 1965); a bromophenol exuded by the red alga *Rhodomela larix* (Phillips and Towers, 1982); diterpenes extracted from *Dictyota menstrualis*, which inhibit settlement and development of a fouling bryozoans (Schmitt et al., 1995); halogenated furanones from *Delisea pulchra* demonstrated broad spectrum antifouling effect (de Nys et al., 1995); and zosteric acid from the seagrass *Zostera marina* inhibited settlement of *Ulva* spores (Shin, 1998). Some waterborne algal compounds also deter larval settlement (Walters et al., 1996), while several antifouling compounds from marine invertebrates have been identified using barnacle larvae (Miki et al., 1996) or mussel bioassay (Devi et al., 1998). Thus, the aim of this study was to find new antifouling substances from seaweeds, sponge and cashew nut oil extracts against fouling bacteria.

MATERIALS AND METHODS

Collection site

Samples of seaweeds, *S. wighiti, Ulva lactate* and sponge *Desmospongiae* sp., were collected by hand picking during low tide near Tuticorin (Lat. 8° 48' 36'' N; Long. 78° 8' 24'' E), Southeast coast of India during January, 2008. Sampled material was transferred to dark recipients and stored in isothermal boxes to prevent photo and thermal degradation during the transport to the laboratory. Wet algae were cleaned from sediments and associated organisms (but no special treatment was employed to remove microorganisms) and divided into two aliquots: one for taxonomic identification (preserved in 4% formalin in seawater) and other to be used for extraction after weighing and dry the sample. This portion was dried at a room temperature of 17°C and in the dark until a steady weight was obtained (dry weight).

Extraction

Seaweed and Sponge were identified, washed with tap water and removed the surface associated materials before extraction. The surface microflora was removed by washing the algal samples for 10 min with 30% ethanol followed by Hellio et al. (2004).

Seaweeds *S. wighiti* and *U. lactate* were shade dried completely for 3-7 days at room temperature and then ground to powder using an electrical blinder. 500 g of coarsely powdered algal material was vortexed and soaked in 5000 ml methanol in a ratio of 1:10 for seven days at 35°C on a shaker at 120 rpm. After one week, algal material was collected and re-extracted with methanol in 3 L capacity round bottom flask in a water bath at 60°C for about 3 h. The individual crude extracts were pooled and filtered through whatman no 1 filter paper fitted with a Buchner funnel using suction pressure followed by the centrifugation (Eppendorf) at 5000 rpm for ten minutes. The supernatant was reduced to a dark green oily natured crude mass in a rotary vaccumn evaporator (Yamato) at 40°C. The resultant extractives were collected in air-tight plastic vials and stored in the refrigerator for further activity studies (Hellio et al., 2004). Sponge sample (50 g wet weight) was cut into small pieces, homogenized and allowed to stand in a dark chamber with a combination of methanol, acetone, n-hexane (1:3:1 v/v/v), extracted for 48 h at room temperature and filtered through Whatman No.1. After that, each sponge extract was evaporated at reduced pressure. The crude extract was stored at 4°C for further analysis.

Antifouling assay

Screening for antifouling assay was performed using algal extracts at a concentration of 30 µg/ml. All assays were done in triplicate. Negative controls with the solvent carrier (5% DMSO v/v) were performed in every assay and showed no inhibition of the biological activities. In all our different assays, we used the activities of cupric sulfate (CuSO4), at the concentration of 1 mg/ml as the controls (Hellio et al., 2004).

Antifouling bacterial tests

Marine fouling bacteria were isolated from the culture collection, which had been collected from the three different test panels (Aluminium, Fibre and Wood) in Vellar estuary. Antifouling bacterial assay testing of the extracts were performed by modified standard disc diffusion method Devi et al. (1997) as previously described by Hellio et al. (2004). A sample consisting of 30 µl/disc of extractwas loaded onto paper disks (6-mm diameter). The microorganism cultures were grown in Zobell Marine Agar 2216, Himedia, Mumbai and 0.1 ml samples of the culture (106 cfu/ml) were spread over the agar. After incubation for 4 days at 20°C, the activity was evaluated by measuring the diameter (in millimeters) of the inhibition zones around the discs.

Mussel bioassay

Antifouling activity against Perna viridis

Juvenile mussels (*Perna viridis,* size 0.5 to 5 cm) were collected during low tide from the Vellar estuary, Parangipettai and kept in a 230 L recirculating laboratory aquarium at a constant temperature (20°C), salinity (35‰) and continued aeration for 12 h. Individuals were disaggregated by carefully cutting the byssus threads, and divided into size groups according to total shell length, ranging from 0.5 to 5 cm in a plastic tray with seawater. Individuals exhibiting substrate exploring behaviour (actively exposing their foot and crawling) were selected for experiments. Antifouling activity was measured by the procedure of Ina et al. (1989) and Goto et al. (1992) being modified. The water-resistant filter paper was cut into 9 cm diameter circles and soaked in solvent (control filter). Another 9 cm diameter set of filter papers (treatment filters) were cut in a chess board pattern (1 cm squares) and soaked in a natural concentration of extracts (determined as the extract equivalent to the DW of alga = DW of filter paper) or in a 15 mM solution of $CuSO_4$ (positive control). All filter paper circles were allowed to air dry. The entire filter circles were placed in the bottom of sterile polystyrene Petri dishes, over which treated chess board filters were placed. Dishes were filled with 80 ml of seawater and three mussel specimens (2.0 - 3.0 cm length) were added. In this way, mussels would have the same area of treated (superior and

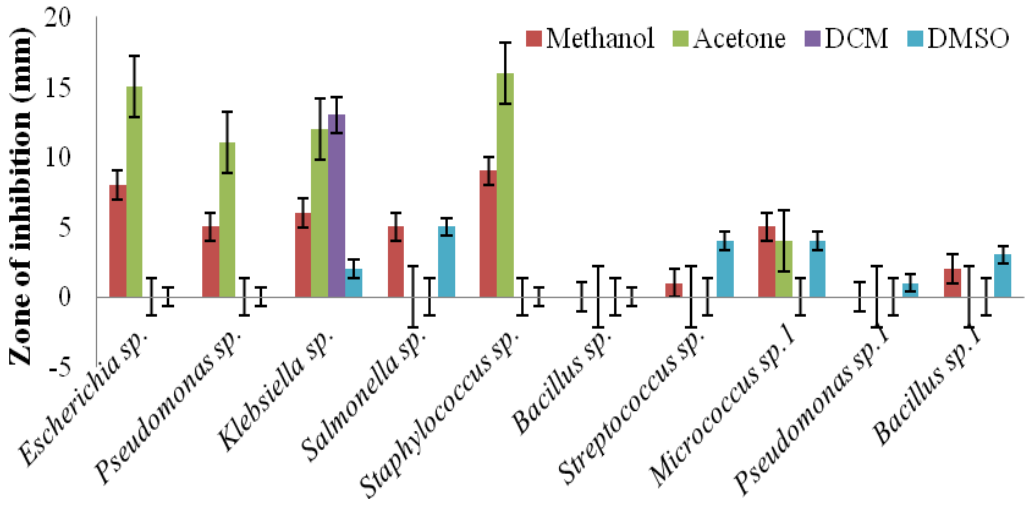

Figure 1. Antibacterial activity of Seaweed *U. lactuca* extracts against biofilm bacteria.

squared) and control (inferior and entire) filter paper to which to attach. Three replicates of each treatment (blank control, positive control- $CuSO_4$, Seaweed and sponge extracts) were used.

Experimental dishes were kept in total darkness, as mussels have been shown to produce more byssal threads when held in the dark (Davis and Moreno, 1995). Experiments were allowed to run for 12 h. Mussel activities were recorded immediately after the start of the experiment, after 2 h and then after 12 h. The activities recorded were number of byssal threads attached to each substratum (control or treated filter paper, shell of another mussel or border of Petri dish). After the 12 h period, all records of attachment were checked, mussels were placed in plastic mesh bags tagged according to treatment, and suspended in a sea aquarium for 24 h to check for possible mortality due to exposure to the test substances.

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) using statistical package of social science (SPSS) version 16.0 for windows. The values are mean ± SE for three experiments in each group.

RESULTS

Antifouling assay

Ten biofilm bacterial strains were isolated from wood, fiber glass and aluminium test panels placed in vellar estuary. The methanol, acetone, dichloromethane (DCM) and DMSO extracts of seaweeds *S. wightii* and *U. lactuca* and cashew nut oil extracts showed antifouling activity against ten bacterial species.

Antibacterial (fouling) assay

Antibacterial activity of seaweed extracts

The sea weed *U. lactuca* extracts proved active against biofilm forming bacteria. The MeOH extract proved to be active against eight species. The maximum inhibition zone formation was 9 mm in *Staphylococcus* sp. and minimum was 1 mm in *Streptococcus* sp. In Acetone extracts, the maximum inhibition zone 16 mm was formed in *Staphylococcus* sp. and minimum (4 mm) was shown in *Micrococcus* sp1. In DCM extract, maximum of 13 mm was shown in *Klebsiella* sp. and no inhibition zone formation was observed in other strains. In DMSO extract, the maximum inhibition zone was formed against *Salmonella* sp. and minimum inhibition zone was found in *Pseudomonas* sp. (Figure 1).

Antifouling bacterial activity of *S. wightii* was active against all 10 fouling bacteria species. The MeOH extract showed activity against eight species. The maximum zone of inhibition showed against *Pseudomonas* sp. (8 mm) and minimum showed against *Staphylococcus* sp. (1 mm). With respect to acetone extracts, the maximum inhibition zone 13 mm was formed in *Salmonella* sp. and minimum of 1 mm was showed in *Pseudomonas* sp. In DCM extract, the maximum 14 mm showed in *Pseudomonas* sp. and 2 mm inhibition zone formation in *Pseudomonas* sp.1 (Figure 2).

Antibacterial activity of sponge and cashew nut oil extracts

The crude extract of cashew nut oil was inhibited the growth of biofilm bacterial strains at a concentration of 30 µg/disc concentration (Figure 3). The negative solvent control (MeOH) had no effect on bacterial growth, while all bacteria were susceptible to the positive controls ($CuSO_4$). The maximum inhibition zone exhibited 8 and 6 mm in methanol and DMSO extract against *Escherichia* sp. and minimum inhibition zone was 2 mm in both acetone and DMSO extracts against *Pseudomonas* sp.

In sponge, *Desmospongiae* sp. showed the maximum

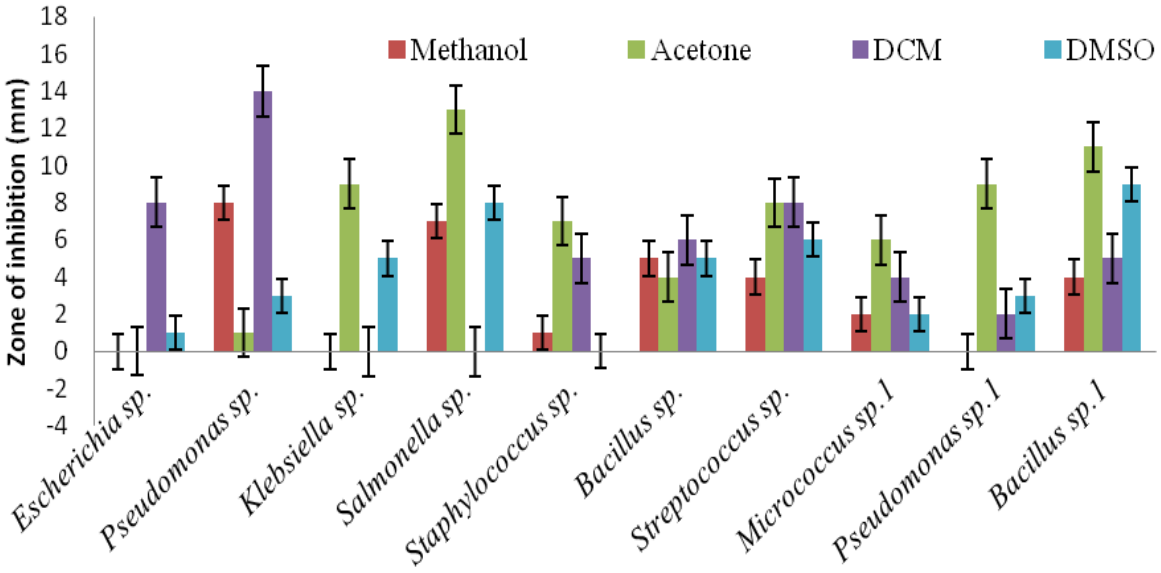

Figure 2. Antibacterial activity of Seaweed *S. wightii* extracts against biofilm bacteria.

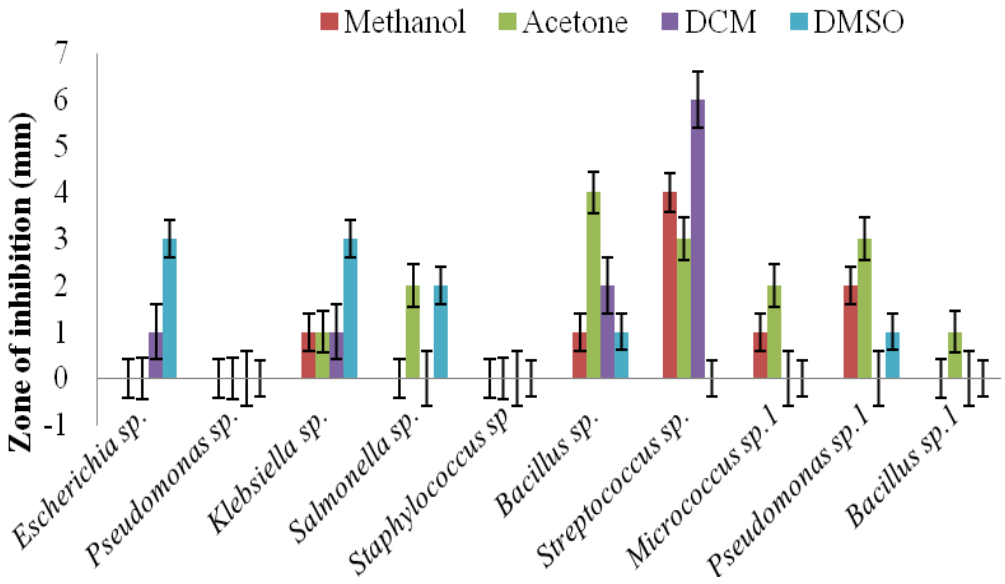

Figure 3. Antibacterial activity of sponge, *Desmosponge* sp. extracts against biofilm bacteria.

zone of inhibition against Escherichia sp., (3 mm) and *Klebsiella* sp. (3 mm) in DMSO extracts. The minimum inhibition zone was found as 1 mm in all three extracts against Klebsiella sp. (Figure 4).

Mussel bioassay

Byssus thread bioassay

Juvenile mussels could possibly be attached to three different types of substrata within Petri dishes: filter paper (control or treated), inner border of the dish or the shell of another mussel (generally the preferred substrate in this gregarious organism). Differences in attachment preferences within treatments were not considered for the purpose of this work, but differences among treatments were taken into account. The mean numbers of attached byssal threads per treatment are presented in Figure 5. Byssal threads were counted in all five treatments (control + two algal + 1 sponge and 1 plant extracts). Mussels attached a mean of 21.6±1.2 byssal threads in controls, which was slight in *S. wightii* (15 ± 0.8), and *U.*

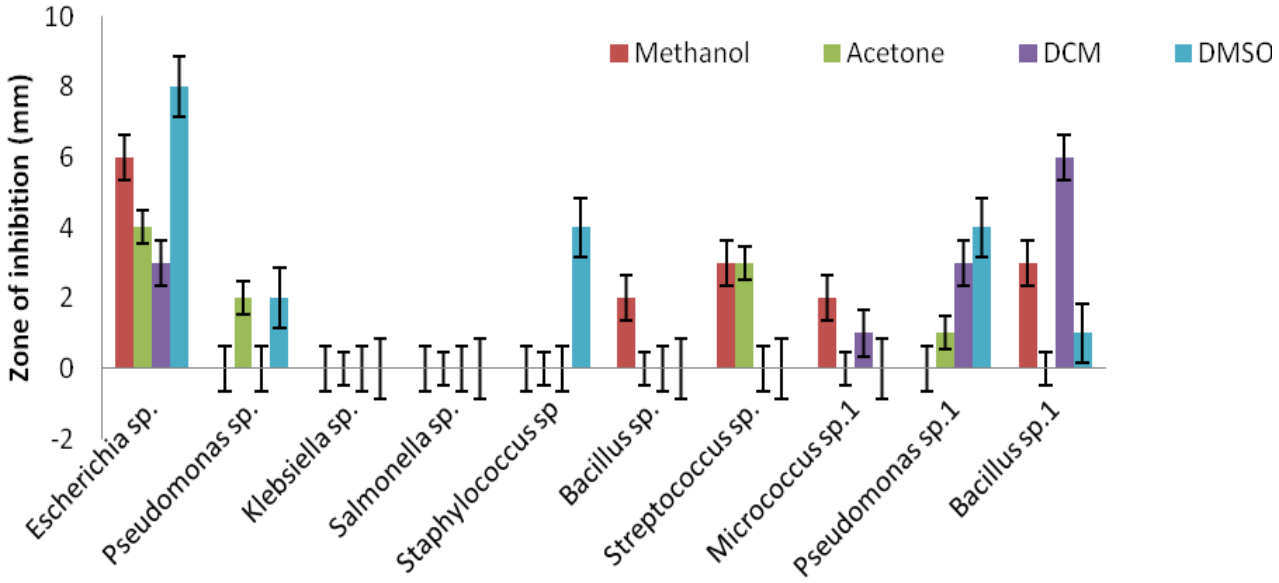

Figure 4. Antibacterial activity of cashew nut oil, *Anacardium occidentale* extracts against biofilm bacteria

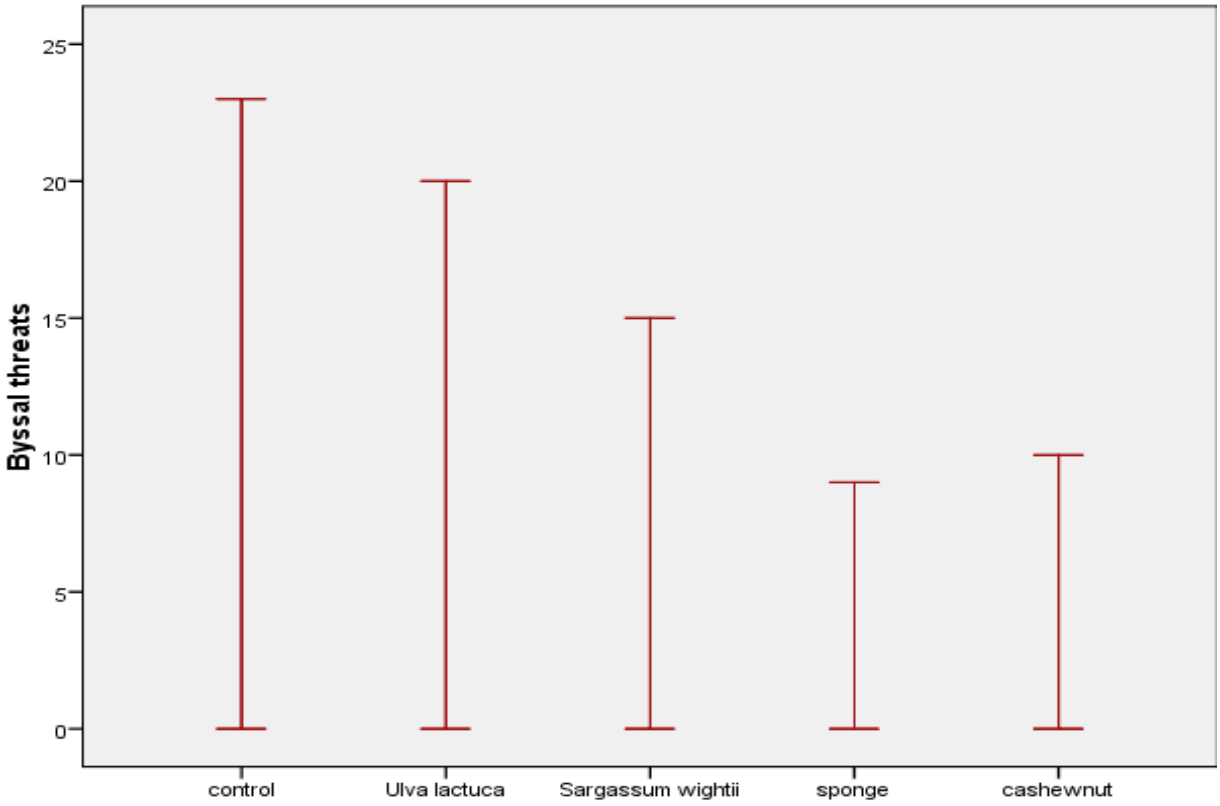

Figure 5. Mean number of attached byssal threads produced by the mussel, *Perna viridis* in response to extracts and compared to controls.

lactuca (20 ± 0.8). The extracts of sponge, D*esmosponge* sp. and cashew nut oil extracts significantly inhibited mussel adhesion (6.0 ± 1.7 and 10.6 ± 0.9) respectively. As a general trend, all extracts inhibited mussel

attachment to some degree.

DISCUSSION

Studies on the antifouling mechanisms utilised by sessile aquatic organisms may provide valuable information for fouling control in marine biotechnology (Hellio et al., 2001a). Antifouling agents derived from natural products may be less environmentally harmful than the current toxins, having less activity against non-target species (Hellio et al., 2000). The results of the antifouling screening of the present study are on the extracts of *U. lactuca* activity against all species of fouling bacterial strains. The highest zone of inhibition was 16 mm against *Staphylococcus* sp. of acetone extract followed by 13 mm against *Klebsiella* sp. of dichloromethane extract. The methanol extracts showed 8 mm zone of inhibition against *Pseudomonas* sp. In the present investigation, *U. lactuca* and *Sargassum* sp. showed potential antifouling activity against biofilm forming bacteria. The present results were closely related to previous studies (Hellio et al., 2000a, b, 2001a, b, 2002; Hallio et al., 2004), which were based on the screening of antifouling activities among 30 macroalgae collected in Brittany coast which good activity against fouling diatoms and bacterial strains.

In India, 37 marine species including 16 species of flora and 21 species of marine fauna was screened as the antifouling potential against fouling organisms by Bhosale et al. (2002). Prabha Devi et al. (1997) reported that some marine plants possess antibacterial properties against the same bacterial strains. Macroalgae produce a wide range of secondary metabolites, many of which exhibit a broad spectrum of bioactivity (Da Gama et al., 2002) and could potentially be used to develop new antifouling agents (Rittschof, 2000; De Nys and Steinberg, 2002). Cho et al. (2001) have tested the antifouling activity of 27 species of common seaweeds which were collected from Korean coast. In previous study, it was reported that methanol extracts of *I. sinicola* at 200 µl ml^{-1} showed a growth inhibition of 0.3 mm. Similar results were obtained in various screening programmes performed in different seas (Padamakumar and Ayyakannu, 1997; Devi et al., 1997; Pawlik, 2000; Bhosle et al., 2002; Da Gamma et al., 2002).

The sponge is a rich source for several alkaloids and peptides exhibiting diverse biological properties such as cytotoxic, antifungal, antimalarial, antituberculosis and antifouling properties (Orabi et al., 2002; Rittschof et al ., 2003; Liu et al., 2004; Raveendran et al., 2008; Limna Mol et al., 2009). The soft coral *Dentronephthya* sp. can control macrofouling on its surface by the production of secondary metabolites (Wilsanand et al., 1999; Dobretsov et al., 2004). Dobretsov et al. (2005) have investigated the antifouling metabolites; demonstrated the antifouling activity of micro and macrofouling communities *in situ*.

All over the world, efforts are oriented towards isolation of eco-friendly antifouling toxins from marine plants and organisms. Consequently few compounds having anti-fouling properties have been identified from marine plants and organisms by number of workers in the past. However, little attention is paid towards terrestrial plants. In light of this, the cashew nut oils are selected for screening of the antifouling activity. In the present study, different solvents extracts of methanol, acetone, DMSO and dichloromethane were used for test against the fouling bacteria. The methanol and ethanol extracts showed promising antifouling activity against the fouling strains. Previously, Swant and Wagh (1997) reported the terrestrial plants *Acacia pennata* and *Barringtonia acutangula* tested against the four fouling diatoms and barnacle larvae.

The other antifouling activity of mussel byssal threat bioassay has produced significant activity against green mussel *P. viridis*. Byssal threat bioassay was performed in two species of algae *S. wightii* and *U. lactuca*, one species of *Desmosponge* sp. and one species of cashew nut oil extracts. Mussels attached a mean of 21.6±1.2 byssal threads in controls, with a slight one in *S. wightii* (15 ± 0.8), and *U. lactuca* (20 ± 0.8). The extracts of sponge, *Desmosponge* sp. and cashew nut oil extracts significantly inhibited mussel adhesion (6.0 ± 1.7 and 10.6 ± 0.9) respectively. In earlier studies, *I. sinicola*, which produced a reaction against the mussel assays, was soluble in acetonitrile, dimethylsulfoxide, ethylacetate, isopropanol and methanol, and isolation of the active blue alkaloid compound is in progress. *Scytosiphon lomentaria* showed antifouling activity only with the mussel. These seaweeds may have species-specific or reaction site-specific substances. The narrow spectral antifouling activity of natural nontoxic antifoulants may be overcome by employing mixtures of several compounds that could cover a range of antifoulant mechanisms (Cho et al., 2001).

Marine sponges contain a large number of compounds that show antibacterial (Walker et al., 1985) and antilarval (Becerro et al., 1997; Lee and Qian, 2003) activity in laboratory bioassays. In the present investigation, the antibacterial activity of sponge *Desmosponge* sp. showed promising activity against fouling bacteria which were isolated from test panels. The maximum inhibition zone showed 3 mm in DMSO extracts against *Escherichia* sp., and *Klebsiella* sp. The minimum inhibition zone was found in 1 mm in all three extracts against *Klebsiella* sp. In earlier studies, Lima Mol et al. (2009) guided purification of the acetone extract of the marine sponge, *Haliclona exigua*, (Gulf of Manners, India) yielded a fraction rich in bis-1-oxaquinolizidine alkaloids, active against seven strains of fouling bacteria as well as cyprids of the cosmopolitan barnacle, *Balanus amphitrite.*

Raveendran and Limna Mol (2009) proposed Natural Product Antifoulants (NPAs) as one of the best replacement options for the most successful antifouling agent, tri-*n*-butyl tin (TBT), which, due to its ecological incompatibility, is currently facing total global ban imposed by International Maritime Organization (IMO).

Conclusion

The strategy adopted in this study has identified a number of seaweed sponge that are capable of producing metabolites that, when incorporated into paint, retain their antifouling activity. Broad spectrum antifouling activity was achieved by combinations of these simple antimicrobial compounds. This work demonstrates the potential of marine algae, sponge and cashew nut oil in the production of antifouling coatings based on biodegradable natural products rather than the toxic compounds in current use.

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Full Length Research Paper

High production of fructooligosaccharides by levansucrase from *Bacillus subtilis* natto CCT 7712

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Fructooligosaccharides (FOSs) are fructose oligomers known as prebiotics that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improving host's health. FOS can be produced by the action of fructosyltranferases from microorganisms, including the *Bacillus subtilis* natto. The objective of the present study was applying response surface methodology in order to optimize the FOS production by levansucrase from *B. subtilis* natto CCT 771. The variables evaluated were sucrose concentration, pH and temperature. From the analysis of the results, the significant variables were pH and sucrose concentration ($p < 0.05$). Levansucrase of *B. subtilis* natto synthesized fructooligosaccharides in all experimental conditions, showing its potential for use in an industrial process of producing FOS from sucrose. The use of response surface methodology allowed the determination of the concentration of sucrose (334 $g \cdot L^{-1}$), pH (6.0) and temperature (45.8°C) for a maximum yield of 54.86 $g \cdot L^{-1}$ FOS.

Key words: Fructooligosaccharides, *Bacillus subtilis*, levansucrase, prebiotics.

INTRODUCTION

Fructooligosaccharides (FOSs) are low molecular weight carbohydrates containing fructose residues with a degree of polymerization from 3 to 9; fructosyl units are bound at the β-(2 → 1) position of sucrose. FOSs are mainly composed of 1-kestose (GF2), nystose (GF3) and fructofuranosyl nystose (GF4) (Yun, 1996). FOSs are part of non-digestible fibers and they act as prebiotics. In the digestive system they serve as a carbon source for colon bacteria, such as lactobacilli and bifidobacteria, which are beneficial to human health (Matulová et al., 2011). The growth and stimulated activity of these bacteria act in the removal of potentially pathogenic bacteria (Gibson and Roberfroid, 1995; Yun, 1996). During microorganism growth, short-chain fatty acids (SCFAs) are produced

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as the end products of oligosaccharide fermentation. SCFAs stimulate the growth of colorectal mucosal cells, retard mucosal atrophy, and decrease the risk of malignant transformation in the colon (Ningegowda and Gurudutt, 2011). Other important properties of FOS are that they have low caloric value, effects in reducing phospholipid, triglycerides and cholesterol levels and help the absorption of calcium and magnesium in gut (Mussato and Teixeira, 2010).

FOS can be obtained from inulin hydrolysis by acid treatment at high temperatures which is expensive and makes it worthwhile to search for new ways of obtaining FOS by either microbial synthesis or enzymatic means (Nemukula et al., 2009). FOSs were produced by the action of fructosyltransferases present in plants and microorganisms (Sánchez et al., 2008). Several microorganisms have β-fructofuranosidases (EC 3.2.1.26) which are responsible for producing FOS obtained from sucrose, such as *Aspergillus oryzae*, *Aureobasidium pullulans*, *Bacillus subtilis* and *Zymomonas mobilis* (Bekers et al., 2002; Euzenat et al., 1996; Sangeetha et al., 2004; Shin et al. 2004). Among them, *Bacillus subtilis* has been the most extensively studied for production of levansucrase, and strains producing high levels of constitutive levansucrase have been isolated. When incubated with sucrose, *B. subtilis* levansucrase catalyses the formation of high and low molecular weight levans. *B. subtilis* natto has shown to have capacity to produce levan by levansucrase extracellular, in sucrose medium and to be able to synthesize FOS by the action of this enzyme (Abdel-Fattah et al., 2005).

Some authors have focused in FOS production by microorganism and its respective enzymes, for example B. circulans (El-Refai et al. 2009), B. subtilis 33 NRC (Abdel-Fattah et al., 2005), B.subtilis C4 (Euzenat et al. 1997), and *Zymomonas mobilis* (Bekers et al., 2002). These authors point out the influence of parameters such as sucrose concentration and temperature in the production and size of the oligofructose chains. Increasing the reaction temperature interferes with FOS synthesis because it accelerates the transfructosylation rate converting 1-kestose rapidly to nystose (Vega-Zuniga and Hansen 2011). Abdel-Fattah et al. (2009) studied the effect of sucrose concentration, temperature and found that increasing the sucrose concentration to 40% favored the formation of FOS and temperature increase was accompanied by a reduction in the oligofructose molecular weight. Other factors such as pH, enzyme concentration and reaction time are parameters that act in FOS production (Sangeetha et al., 2005).

There are many factors that influence FOS production as temperature, pH, medium composition, agitation and time of cultivation. An alternative is the use of response surface methodology, a method used in the optimization of the composition of media and other critical variables responsible for biomolecule production. This method allows testing multiple variables with a minimum number of experiments and evaluates the interaction among factors (Liu et al. 2010).

In this work, response surface methodology was used as an optimization tool for the production of fructooligosaccharides by levansucrase from *Bacillus subtilis* natto CCT 7712. Three independent variables, pH, temperature and concentration of sucrose were tested in the reaction medium.

MATERIALS AND METHODS

Microorganisms

B. subtilis natto CCT 7712 was maintained at 4°C on slant medium containing ($g \cdot L^{-1}$): peptone 50, yeast extract 30 and agar 20. This strain was isolated from soybeans fermented, a Japanese food called “natto” at the Department of Biochemistry and Biotechnology of State University of Londrina (Brazil) and identified by Fundação André Tosello (Campinas- Brazil). The product “natto” was purchased at a health food store. The soybeans were macerated and were diluted with distilled water. Serial dilutions were made, inoculated on Petri dishes containing standard medium and incubated at 37.5°C for 48 h.

Crude enzyme production

First stage of enzyme production was cultivation of the microorganism source of levansucrase. *B. subtilis* was activated in inoculum medium with the following composition: 100 $g \cdot L^{-1}$ of sucrose, 2 $g \cdot L^{-1}$ of yeast extract, 2 $g \cdot L^{-1}$ of KH_2PO_4, 1 $g \cdot L^{-1}$ of $(NH_4)_2SO_4$ and 0.5 $g \cdot L^{-1}$ of $MgSO_4.7H_2O$.

The second step was the fermentation, this medium contained 300 $g \cdot L^{-1}$ of sucrose, 2 $g \cdot L^{-1}$ of yeast extract, 1 $g \cdot L^{-1}$ of KH_2PO_4, 3 $g \cdot L^{-1}$ of $(NH_4)_2SO_4$, 0.6 $g \cdot L^{-1}$ of $MgSO_4.7H_2O$, 0.2 $g \cdot L^{-1}$ of $MnSO_4$, H_2O and 0.25 $g \cdot L^{-1}$ of ammonium citrate. The pH was adjusted to 7.7; the culture was incubated at 37°C in a rotary shaker at 150 rpm for 48 h. The cells obtained in the first stage were used as an inoculum to fermentation medium, were transferred 0.2 g of cells for each liter of fermentation medium. At the end of the incubation period, the culture was centrifuged (4°C, 9056 x *g*) using a refrigerated centrifuge (Hetic V 320R), and the supernatant and the cells were separated. The supernatant was considered as source of levansucrase.

Enzyme assay

Levansucrase enzyme was assayed by estimating the reducing sugar released during exopolymer hydrolysis. The reaction mixture, containing 250 µL enzyme extract and 250 µL 1 M sucrose in acetate buffer (pH 5.0) was incubated at 30°C for 2 h (Ananthalakshmy and Gunasekaran 1999). After 2 h, the exopolysaccharide was precipitated by adding chilled ethanol. The precipitate was hydrolyzed in 1 mL 1 M HCl at 100°C for 1 h, the solution was then neutralized by adding NaOH 2 M and the reducing sugars were determined by the Somogyi (1952) and Nelson (1944) methods.

One unit of activity (UA) was defined as the amount of enzyme that released 1 µMol reducing sugar per mL per minute under the experimental conditions. All experiments used standardized crude enzyme extract at 6 U.

Table 1. Fructooligosaccharide production using levansucrase of B. *subtilis* natto with three independent variables at three levels after 12 h reaction time and enzymatic activity = 6 u/ml.

Run	x_1 (X_1, $g \cdot L^{-1}$)	x_2 (pH)	x_3 (X_3, °C)	FOS ($g \cdot L^{-1}$)
1	-1 (150)	-1 (5)	-1 (45)	47,86
2	-1 (150)	-1 (5)	1 (55)	22,12
3	-1 (150)	1 (7)	-1 (45)	37,62
4	-1 (150)	1 (7)	1 (55)	30,69
5	1 (350)	-1 (5)	-1 (45)	50,53
6	1 (350)	-1 (5)	1 (55)	39,32
7	1 (350)	1 (7)	-1 (45)	48,08
8	1 (350)	1 (7)	1 (55)	33,69
9	-1,68 (81,8)	0 (6)	0 (50)	40,31
10	1,68 (418,18)	0 (6)	0 (50)	67,73
11	0 (250)	-1,68 (4,3)	0 (50)	22,55
12	0 (250)	1,68 (7,7)	0 (50)	18,57
13	0 (250)	0 (6)	-1,68 (41,6)	57,99
14	0 (250)	0 (6)	1,68 (58,4)	53,49
15	0 (250)	0 (6)	0 (50)	51,15
16	0 (250)	0 (6)	0 (50)	64,21
17	0 (250)	0 (6)	0 (50)	54,38
18	0 (250)	0 (6)	0 (50)	59,38
19	0 (250)	0 (6)	0 (50)	60,40

X1: Sucrose concentration, X2: pH and X3: temperature.

FOS enzymatic synthesis using response surface methodology

Response surface methodology with a central composite design was used to determine the optimal condition of sucrose concentration (X_1), pH (X_2) and temperature (X_3) for maximal FOS formation (Table 1). The variables were coded as in Equation 1 1:

$$x_i=(X_i-X_{cp})\Delta X_i \quad (1)$$

Where, x_i is the coded level of the variable, X_i is the real level of the variable, X_{cp} is the real level of the variable at the center point, and ΔX_i is the step change value in the real level. The parameters that were simultaneously varied for FOS production in the response surface methodology (RSM) were sucrose concentration (81.8 $g \cdot L^{-1}$ to 418.18 $g \cdot L^{-1}$), pH (4.3 - 7.7) and temperature (41.6 to 58.4°C). The optimal response of the FOS formation was predicted by the following quadratic Equation 2:

$$\hat{Y} = b_0 + \sum b_i x_i + \sum b_{ii} x^2_{ii} + \sum b_{ij} x_i x_j \quad (2)$$

Where, $\hat{Y}$ is the response variable, b_0 is the constant, b_i is the coefficient for the linear effect , b_{ii} is the coefficient for the quadratic effect, b_{ij} is the coefficient for the interaction effect, and x is the coded level of the variable.

The experiments were performed in 1.5 mL of standardized sucrose solution in 0.1 M citrate buffer or 0.1 M phosphate buffer with the addition of 0.5 mL crude enzyme extract. The reaction was carried out in a water bath for 12 h. After stopping the reaction by incubating the samples in a boiling water bath for 15 min, the FOS production was analyzed by high-performance liquid chromatography - HPLC (Sageentha et al., 2004). Sucrose concentration, pH and temperature were studied.

Analytical procedures

FOS production was analyzed with HPLC using Shimadzu equipment with a Shimadzu RID-10A refractive index detector. The column used was AMINEX Carbohydrate HPX-87C (300 mm x 7.8 mm Biorad). The column temperature was maintained at 80°C. The samples (20 µL) were eluted with 0.6 $mL \cdot min^{-1}$ Milli-Q water and the FOS standards 1-kestose (GF_2 - 504.44 Da) and 1- nystose (GF_3 - 666.58 Da) were from Sigma-Aldrich. The total FOS production was calculated as the sum of 1-kestose and 1-nystose expressed in $g \cdot L^{-1}$.

Statistical and data analysis

All statistical analyses were conducted using STATISTICA (data analysis software system), Version 7.0 (StatSoft, Inc. 2004, EUA). Differences were considered significant at p-values < 0.05.

RESULTS

Nineteen assays were performed to define the best condition for enzymatic synthesis of FOS. Total FOS produced was analyzed by HPLC, in the chromatograms obtain from aliquots of the reactions.

There were five peaks which correspond to fructose, glucose, sucrose and two types of FOS (1-kestose and 1-nystose). Therefore, it can be concluded that this levansucrase only synthesized small chains of FOS with molecular weight of 556 and 666 Da.

Table 2. Analysis of variance (ANOVA) for the second-order model of levansucrase FOS production.

Factor	Sum of squares	d.f.	Mean square	p-value
Sucrose (L)	462.389	1	462.389	0.0092*
pH (L)	19.79	1	1979	0.5125
Temperature (L)	317.613	1	317.613	0.0231*
Sucrose (Q)	65.389	1	65.389	0.246
pH (Q)	2682.78	1	2682.78	0.000024*
Temperature (Q)	34.116	1	34.116	0.394
x_1x_2	5.136	1	5.136	0.736
x_1x_3	6.224	1	6.224	0.711
x_2x_3	30.531	1	30.531	0.419
Lack of fit	27696	5	55.393	0.248
Pure error	106.218	4	26.554	

R^2=0.9020; *Significant for p-values < 0.05.

The optimum sucrose concentration (X_1), pH (X_2) and temperature (X_3) for maximum FOS formation were investigated by response surface methodology with a central composite factorial design. The results are shown in Tables 1 and 2. Regression analysis of the experimental data shows that the response producing FOS can be predicted by the following second order polynomial equation:

$$Y= 55.3762+5.8187x_1-13.7505x_2^2-4.8225x_3 \quad (3)$$

Where, Y is the response for the FOS production, x_1, x_2 and x_3 are coded values for sucrose, pH and temperature, respectively.

The model coefficient of determination (R^2) was 0.902, which indicates that 90.2% of the variability of the responses can be explained by the model. This value was considered acceptable, because according to Joglekar and May (1990), the coefficient of determination must be at least 80%. The lack of fit was not significant (p = 0.248), indicating that the model equation was suitable for predicting FOS production.

The analysis of the regression equation showed that intercept, the linear term of sucrose, the quadratic term of the temperature and pH were significant at 5% (Table 2). Among the tests performed, the highest FOS production was achieved in the experiment 10. In this point, the FOS production was 67.73 g. L^{-1}, the three variables were set in the following parameters: sucrose concentration of 418.18 $g{\cdot}L^{-1}$, pH 6 and 50°C of temperature. The FOS production significantly decreased to 18.57 $g{\cdot}L^{-1}$ with sucrose at a concentration of 250 $g{\cdot}L^{-1}$, pH 7.7 and 50°C (Table 1; Test 12).

The effect of the sucrose concentration can be seen by comparing the experiments presented at Table 1. In the assay 9, the sucrose content was 81.8 $g{\cdot}L^{-1}$ and in the assay 10, the sucrose content was 418.18 $g{\cdot}L^{-1}$, in both the pH were 6.0 and temperature, 50°C. The yield was 40.31 $g{\cdot}L^{-1}$ in assay 9 and 67.73 $g{\cdot}L^{-1}$ in assay 10, with an increase of about 68 %, indicating a positive effect of this variable, which can be seen in Equation 3.

The temperature was significant for FOS production (p = 0.0231) (Table 2) and the yield was reduced when increasing the temperature from 45 to 55°C for assays 1 to 8 (Table 1). In assay 1, yield was 47.86 $g{\cdot}L^{-1}$ FOS and with an increase of 10°C in experiment 2 it was 22.12 $g{\cdot}L^{-1}$. The optimum pH for FOS production was around 6.0.

Figure 1 shows the response surface for FOS production by levansucrase of *B. subtilis* natto as function of sucrose concentration. Increase in sucrose promoted FOS production while temperature above 45°C reduced the synthesis.

In order to determine the best conditions to synthesis of FOS, a predictive analysis was done. The statistical analysis indicated the theoretical maximum value of 63.62 $g{\cdot}L^{-1}$, which could be obtained setting the variables in the following parameters: sucrose concentration of 334 $g{\cdot}L^{-1}$, pH 6.0 and temperature of 45.8°C (Figure 2). A validation of predictive model (Equation 3) was necessary, therefore four tests were performed using the conditions cited above. The average yield of experiments was 54.86 $g{\cdot}L^{-1}$, which was compared statically with theorical value. The analysis shows that the predicted value and experimental validation were not statistically different at 5% level of significance. The optimization of FOS production shown in this work demonstrates the potential of levansucrase from *B. subtilis* for this application.

DISCUSSION

Recently, many studies have focused on the production of fructooligosaccharides. These works investigated

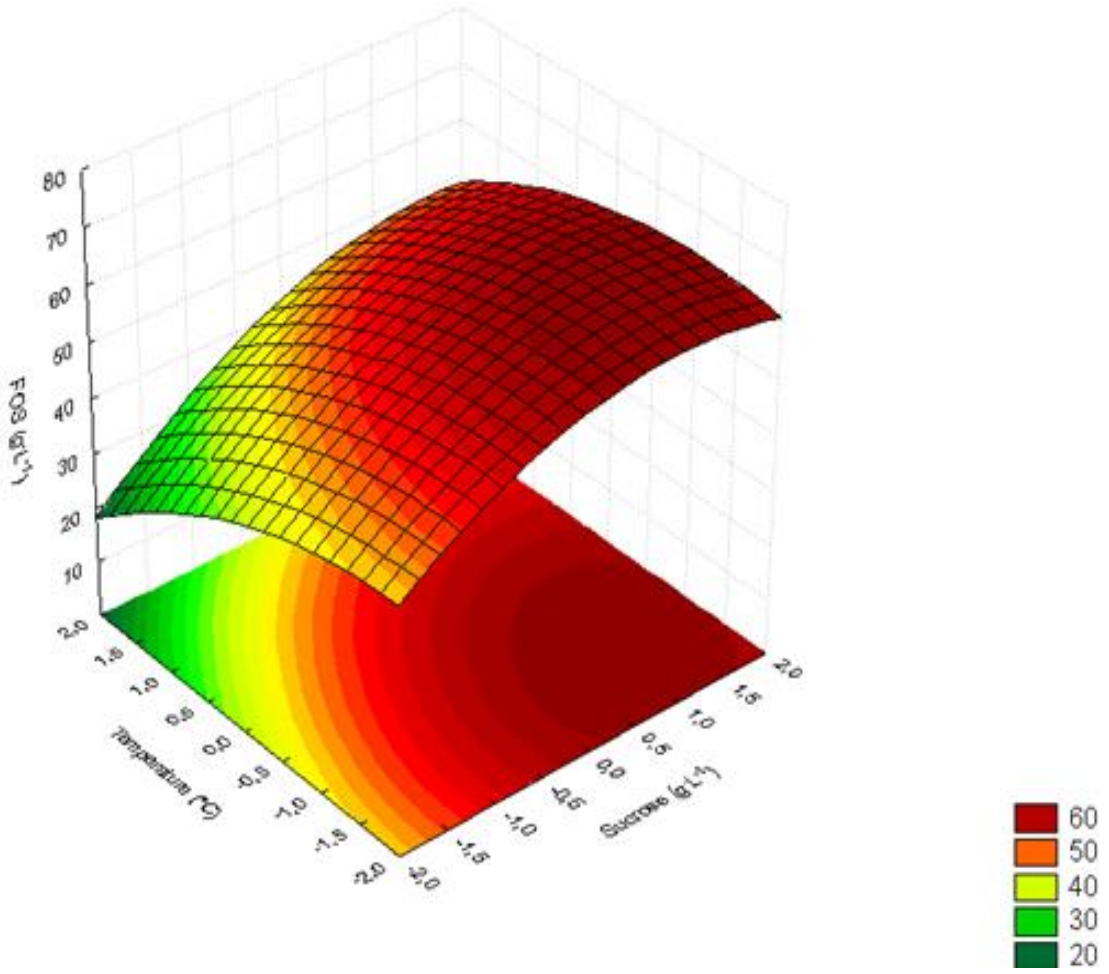

Figure 1. Response surface and contour plots for fructooligosaccharides production by levansucrase of *Bacillus subtilis* natto as a function of sucrose and temperature, pH=6.0.

microbial FOS production in sucrose medium by the action of fructosyltransferases and β-fructofuranosidades from fungi and bacteria (Park et al., 2003). The best FOS productions were obtained in the assays in which the sucrose concentrations were among the highest. Silva et al. (2011) noticed similar effect for fructosyltransferase from Aureobasidium pullulan, the sucrose concentration was positively related to FOS, once the fructosyltransferase and transfructosylation performance more efficiently in media with high concentrations of this substrate.

This positive effect may be due to the fact that activity of levansucrase increases with the increase of sucrose in medium (Belghith et al., 2012). According to Vega-Paulino and Zuniga-Hansen (2012), the initial sucrose concentration is the major factor in FOS formation because it increases the availability of fructosyl acceptors and reduces water availability; so that this condition can improve the formation of FOS. Hettwer and Rudolph (1995) reported that at higher sucrose concentrations, levan production at the beginning is high but later its synthesis is inhibited. Then, the hydrolase activity increases, accumulating oligosaccharides and increasing glucose concentration that continues inhibition of levansucrase. Temperature was an important factor, as soon as it increases; FOS production improves until a specific level, after this specific point the production decreases. Similar behavior was observed for fructosyltransferase from *Aspergillus aculeatus* (Ghazi et al., 2007) and FOS synthesis decreased from 60°C and the catalytic activity was reduced after 2 h of reaction due to thermal inactivation. The levansucrase of *B. subtilis* natto in the present work showed to be more active at 45.8°C.

The pH of medium plays an important role in fructosyltransferase activity. The optimum pH 6.0 verified in this study corresponds to the optimum pH for the activity of the levansucrase enzyme from *Bacillus* sp. described by Ammar et al. (2002). In fact, most of the levansucrases reported so far have showed optimum pH

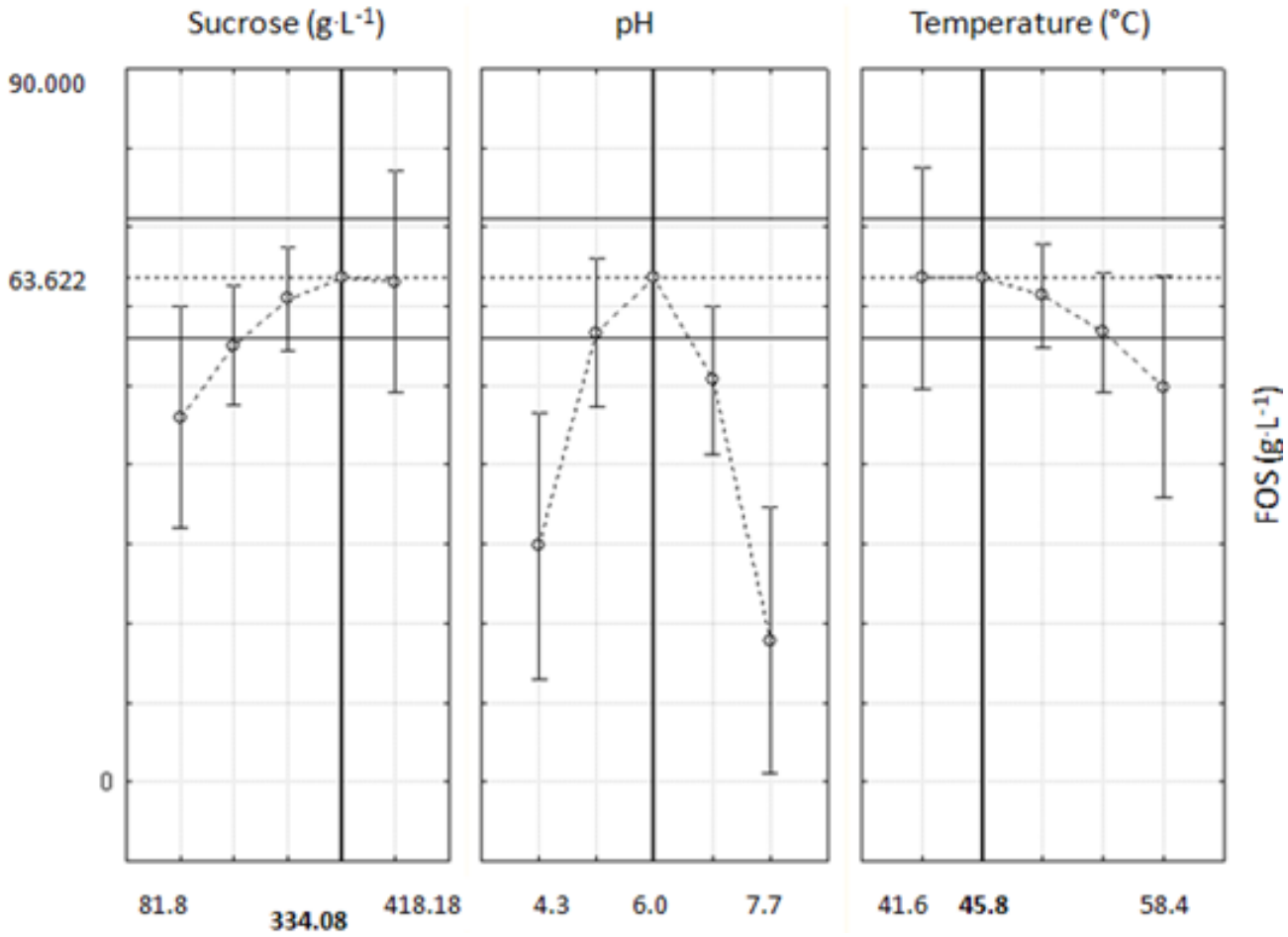

Figure 2. Optimization of the production of fructooligosaccharides . (Statistca Software 7.0).

values ranging between 5.0 and 6.5, as reported by Vega- Paulino and Zúniga (2012).

Levansucrase from *B. subtilis* natto synthesized fructooligosaccharides in all experimental conditions, showing its potential for use in an industrial process of producing FOS from sucrose. The response surface methodology allowed the determination of the best values of sucrose (334 $g.L^{-1}$), pH (6.0) and temperature (45.8°C) for a maximum yield of 54.86 $g.L^{-1}$ of FOS.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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Full Length Research Paper

Response surface method to optimize the low cost medium for protease production using anchovy meal from ascidian associated *Bacillus* sp. GA CAS10

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A protease producing *Bacillus* sp. GA CAS10 was isolated from ascidian *Phallusia arabica*, Tuticorin, Southeast coast of India. Response surface methodology was employed for the optimization of different nutritional and physical factors for the production of protease. Plackett-Burman method was applied to identify important factors (anchovy waste, KH_2PO_4, NaCl and temperature) influencing protease production. Further optimization was done by response surface methodology using central composite design. Under the optimized conditions by central composite design, the protease experimental yield (842.102 U/ml) closely matched the predicted yield by the statistical model (830.307 U/ml) with R^2 = 99.94%. The time course of protease production was increased using the RSM optimized medium (856.29 U/ml) (anchovy waste 20.50 g/l, KH_2PO_4 3.06 g/l, NaCl 42.91 g/l, temperature 43.36°C, 42 h and pH 9) compared with the un-optimized basal medium (267.33 U/ml). The improvement of protease production by microbial conversion of anchovy waste suggested its potential utilization to generate high value added products using cheap carbon and nitrogen substrates.

Key words: Protease, anchovy waste, statistical optimization, ascidian associated bacteria.

INTRODUCTION

Proteases constitute 60 to 65% of the world's industrial enzyme market; most of which are alkaline proteases (Banik and Prakash, 2004). The use of alkaline proteases has increased remarkably in industries such as food, pharmaceutical, leather and textile which are the major consumers of these enzymes (Jellouli et al., 2009). Proteases can be obtained from plants, animals and microbial sources (bacteria, fungi, yeasts and actinomycetes). In these three sources microbial sources are renewable and among the microbial sources, the genus Bacillus has been extensively studied by many researchers. Several *Bacillus* strains such as, *Bacillus*

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Abbreviations: RSM, Response surface methodology; **CCD,** central composite design; **ANOVA,** analysis of variance; **PCR,** polymerase chain reaction.

Table 1. Experimental variables at different levels used for the production of alkaline protease by *Bacillus* sp. GA CAS10 using Plackett-Burman design.

Parameter	Variable	Level		Effect	Coefficient	*t*-Value	p-Value
		+	-				
Constant					211.19	22.09	0.000
A	Anchovy waste	20	1	187.15	93.58	19.03	0.000
B	Fructose	10	1	22.99	11.49	2.34	0.080
C	KCl	5	1	-22.75	-11.37	-2.31	0.082
D	KH_2PO_4	1	0.1	-31.80	-15.90	-3.23	0.032
E	NaCl	30	10	26.31	13.16	2.97	0.046
F	Temperature	40	30	124.93	62.46	12.70	0.000
G	pH	6	9	2.79	1.40	4.26	00.013

clausii (Oskouie et al., 2008), *Bacillus subtilis* (Ghafoor and Hasnain, 2009), *Bacillus sphaericus* (Afify et al., 2009), *Bacillus pseudofirmus* (Sen et al., 2009), *Bacillus aquimaris* (Shivanand and Jayaraman, 2009), *Bacillus circulans* (Subba Rao et al., 2009), *Bacillus licheniformis* (El Hadj-Ali et al., 2010) and *Bacillus cereus* (Shah et al., 2010) are known to produce industrially important proteases and also large proportion of commercially available proteases are currently derived from *Bacillus* strains.

The anchovy fish catch accounts for a major portion of the world's overall fish catch, the majority being used for fish meal production and only a comparatively small amount being used for human consumption. Anchovy meal is known for its nutrient richness, containing 63 to 66% protein and 9.14% lipid, which may help promote excellent microbial growth and protease production (Esakkiraj et al., 2011; Kratzer et al., 1994; Bimbo, 1990; Turan et al., 2007).

Studies related to statistical optimization of medium constituents for protease production using anchovy meal by ascidian associated *Bacillus* sp. are meager and hence the present study was designed to optimize the medium and culture conditions to enhanced protease production by ascidian associated *Bacillus* sp. GA CAS10 using low-cost marine fish waste substrate by response surface methodology.

MATERIALS AND METHODS

Microorganism

A protease producing bacterium GA CAS10 used in this study was isolated from an ascidian *Phallusia arabica*, obtained from Tuticorin, Southeast coast of India, following the method of Sathish Kumar et al. (2014). The strain GA CAS10 produced a clear zone when streaked on skim milk agar after 48 h, and was identified as *Bacillus* sp. based on the morphological and biochemical characteristics (Holt et al., 1994) and also confirmed through molecular characterization. The bacterial genomic DNA was extracted by phenol chloroform method (Marmur, 1961) and 16S rRNA gene was amplified by using forward primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'-GGGCGGTGTGTACAAGGC -3'). Polymerase chain reaction (PCR) was performed under the following conditions; 35 cycles consisting of initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 S, annealing at 55°C for 30 s and followed by final extension of 5 min at 72°C. The 16S rRNA gene sequences were obtained by an automated DNA sequence (Megabace, GE) and homology was analyzed with sequences in the Gen Bank using CLUSTAL X software. The phylogenetic tree was constructed by the neighbor-joining method using software (MEGA 5.0) (Saitou and Nei, 1987).

One factor at a time experiments

Anchovy waste was purchased from the local fish market at Parangipettai, Tamil Nadu, India and powdered well. The strain GA CAS10 was cultured using Zobell marine broth with 2% NaCl. Then, 10% of enriched seed culture was inoculated into a 250 ml flask containing 50 ml basal medium (w/v) (glucose 0.5%, yeast extract 1.0%, potassium dihydrogen orthophosphate 0.1%, sodium chloride 3%, magnesium sulphate 0.3%). The culture was incubated in a shaker at 150 rpm for 48 h at 40°C. The cells were harvested by centrifugation at 10,000 rpm for 15 min and the supernatant was further used for protease assay. Initial screening of the most significant carbon and metal ions to maximize protease production were performed by one-variable-at-a-time approach. Carbon sources such as fructose, lactose, xylose, maltose, starch and mannitol were added individually in the basal medium at a concentration of 0.5 and 0.1% and various metal ions (calcium chloride, magnesium chloride, barium chloride, potassium chloride, zinc chloride and zinc sulphate) were tested for protease production.

Statistical optimization of protease production

Plackett - Burman design

Using Plackett-Burman design is an efficient way to screen the important medium components among a large number of process variables which is required for elevated protease production by screening '*n*' variables in '*n* + 1' experiment. Each factor was examined at two levels: -1 for a low level and +1 for a high level. The variables chosen for the present study were anchovy waste, fructose, KCl, KH_2PO_4, NaCl, temperature and pH (Table 1). Variables were evaluated in 12 experimental trials as shown in Table 2. The design was run in a single block and the order of the experiments was fully randomized. The design was developed by the Minitab package version 16.

Table 2. Plackett-Burman design matrix for seven variables with coded values along with the observed and predicted protease production.

Run order	A (g/l)	B (g/l)	C (g/l)	D (g/l)	E (g/l)	F (g/l)	G (g/l)	Protease production(U/ml)	
								Observed	Predicted
1	+1	-1	+1	-1	-1	-1	+1	296.12	278.052
2	+1	+1	-1	+1	-1	-1	-1	247.21	250.082
3	-1	+1	+1	-1	+1	-1	-1	91.42	98.295
4	+1	-1	+1	+1	-1	+1	-1	317.11	329.273
5	+1	+1	-1	+1	+1	-1	+1	321.17	318.298
6	+1	+1	+1	-1	+1	+1	-1	417.25	410.375
7	-1	+1	+1	+1	-1	+1	+1	209.27	207.015
8	-1	-1	+1	+1	+1	-1	+1	77.25	85.410
9	-1	-1	-1	+1	+1	+1	-1	209.25	191.182
10	+1	-1	-1	-1	+1	+1	+1	439.26	452.040
11	-1	+1	-1	-1	-1	+1	+1	259.31	261.565
12	-1	-1	-1	-1	-1	-1	-1	68.71	71.743

Optimization by central composite design

The next level in the optimization of the medium required to determine the optimal levels of the significant variables in protease production. To this purpose, response surface methodology (RSM) was adopted for the augmentation of total protease production using a central composite design (CCD). The most significant variables were selected as follows: anchovy waste, KH_2PO_4, NaCl and temperature. A total of 31 experiments were formulated using the statistical software package 'Minitab 16.0'. The central values of all variable were coded as zero. The full experimental plan, with regard to their values and the corresponding experimental and predicted responses values (Y) are provided in Table 4. The data obtained from the RSM on protease production were subjected to analysis of variance (ANOVA). The experimental results of RSM were fitted with the response surface regression procedure using the following second order polynomial equation:

$$Y = \beta 0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

Where, *Y* is the predicted response, *Xi* and *Xj* are independent factors, $\beta 0$ is the intercept, βi is the linear coefficient, βii is the quadratic coefficient, and βij is the interaction coefficient. The statistical software package 'Minitab' (Version 16.0) was used to analyze the experimental data.

Time course of protease production

To study the relation between protease production and the growth profile of the bacterium, 50 ml of the optimized production media by response surface methodology was inoculated in 250 ml flasks and the growth was measured at regular intervals by viable count (spread plate method) determination. The protease production at different time intervals was determined using the standard protease assay.

Enzyme assay

The protease activity was measured by incubating the reaction mixture containing 0.2 ml of diluted enzyme and 1.25 ml of 1.25% casein in 50 mM Tris-HCl buffer (pH 9) for 30 min at 37°C. The reaction was terminated by adding 5 ml of 0.19 M trichloroacetic acid. The reaction mixture was centrifuged and the soluble peptides in the supernatant were measured according to the method of Todd (1949) with tyrosine as reference. One unit of protease was defined as the amount of enzyme required to release 1 µg of tyrosine per min.

RESULTS AND DISCUSSION

Microorganism

A protease producing strain GA CAS10 was isolated from the tissue of ascidian *P. arabica*, Tuticorin from Southeast coast of India. The morphological and biochemical characteristics of the strain revealed that the strain is a Gram-positive and endospore forming bacilli (rod) with catalase but without oxidase (Table 3). The phylogenetic analysis (Figure 1) and BLAST search confirmed the isolate as *Bacillus* sp. 16S rRNA sequence of GA CAS10 has been deposited as *Bacillus* sp. GA CAS10 in the GenBank database (GenBank ID: JX627407.1).

Plackett-Burman design

A number of studies have been carried out on optimization of different physicochemical parameters of different organisms for maximum protease production using response surface methodology (Sathish Kumar et al., 2014). In general there is no defined medium designed for the production of alkaline protease from different microbial sources (Gupta et al., 2002). Based on the one-variable-at-a-time approach, protease production by *Bacillus* sp. GA CAS10 revealed fructose as optimum carbon source (267.33 U/ml) (Figure 2a) and potassium chloride as suitable source of metal ions (239.21 U/ml)

Table 3. Morphological and biochemical characteristics of strain *Bacillus* sp. GA CAS10.

Characteristic	Result
Shape	Rod
Gram staining	Positive
Spore formation	-
Motility	+
Glucose	+
Sucrose	+
Glycerol	-
Maltose	+
Starch hydrolysis	-
Gelatin hydrolysis	-
Urease	-
Lipase	-
Casein hydrolysis	+
Amylase	+
Catalase activity	+
Oxidase	+
Indole	+
Citrate	+

Table 4. Experimental conditions in variables of the central composite design and the corresponding experimental responses.

Run order	Anchovy waste (g/l)	KH_2PO_4 (g/l)	NaCl (g/l)	Temperature (^oC)	Protease production (U/ml)	
					Observed	Predicted
1	15.75	2	31.0	42.5	570.28	570.87
2	25.25	2	31.0	42.5	650.26	654.02
3	15.75	4	31.0	42.5	550.09	551.94
4	25.25	4	31.0	42.5	692.45	693.27
5	15.75	2	60.0	42.5	487.45	489.07
6	25.25	2	60.0	42.5	463.20	468.07
7	15.75	4	60.0	42.5	450.26	453.52
8	25.25	4	60.0	42.5	487.24	490.69
9	15.75	2	31.0	47.5	487.26	485.37
10	25.25	2	31.0	47.5	495.11	497.69
11	15.75	4	31.0	47.5	501.42	502.39
12	25.25	4	31.0	47.5	572.94	572.88
13	15.75	2	60.0	47.5	532.65	537.67
14	25.25	2	60.0	47.5	446.11	445.83
15	15.75	4	60.0	47.5	540.26	538.06
16	25.25	4	60.0	47.5	499.15	504.40
17	11.00	3	45.5	45.0	511.17	510.25
18	30.00	3	45.5	45.0	566.24	559.74
19	20.50	1	45.5	45.0	549.13	544.69
20	20.50	5	45.5	45.0	587.31	584.33
21	20.50	3	16.5	45.0	451.20	450.58
22	20.50	3	74.5	45.0	307.11	300.30
23	20.50	3	45.5	40.0	681.29	674.88
24	20.50	3	45.5	50.0	604.10	603.09
25	20.50	3	45.5	45.0	825.19	822.38

Table 4. Contd.

26	20.50	3	45.5	45.0	825.21	822.38
27	20.50	3	45.5	45.0	820.21	822.38
28	20.50	3	45.5	45.0	825.16	822.38
29	20.50	3	45.5	45.0	820.29	822.38
30	20.50	3	45.5	45.0	825.41	822.38
31	20.50	3	45.5	45.0	825.19	822.38

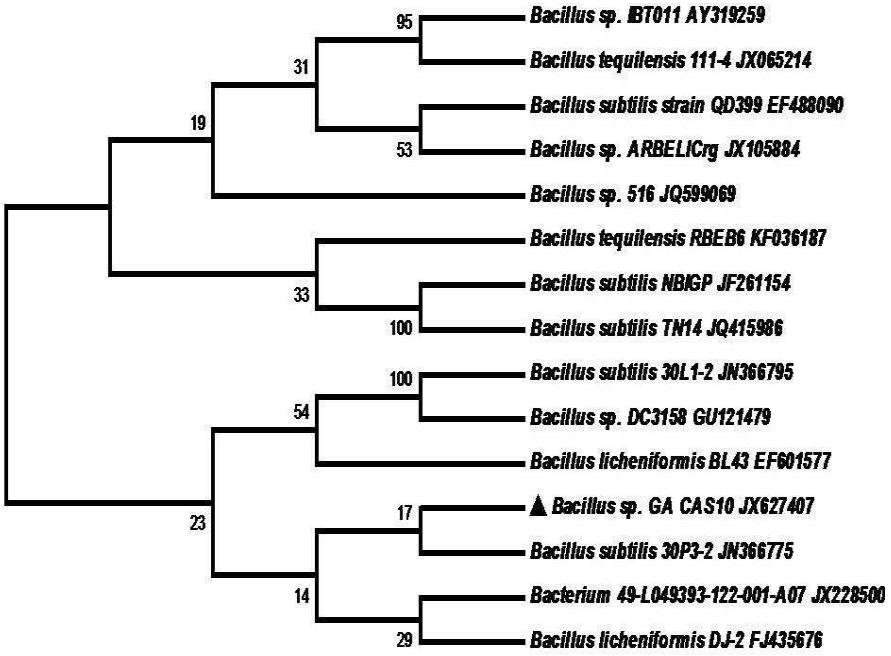

Figure 1. Phylogenetic tree of isolate *Bacillus* sp. GA CAS10 and their closest NCBI (BLAST) strains based on the 16S rRNA gene sequences.

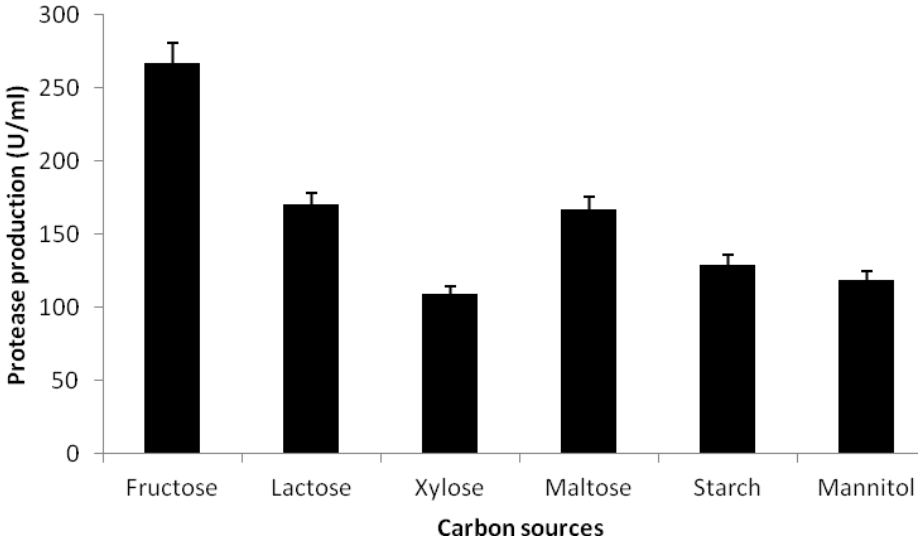

Figure 2a. Effect of different carbon sources on protease production.

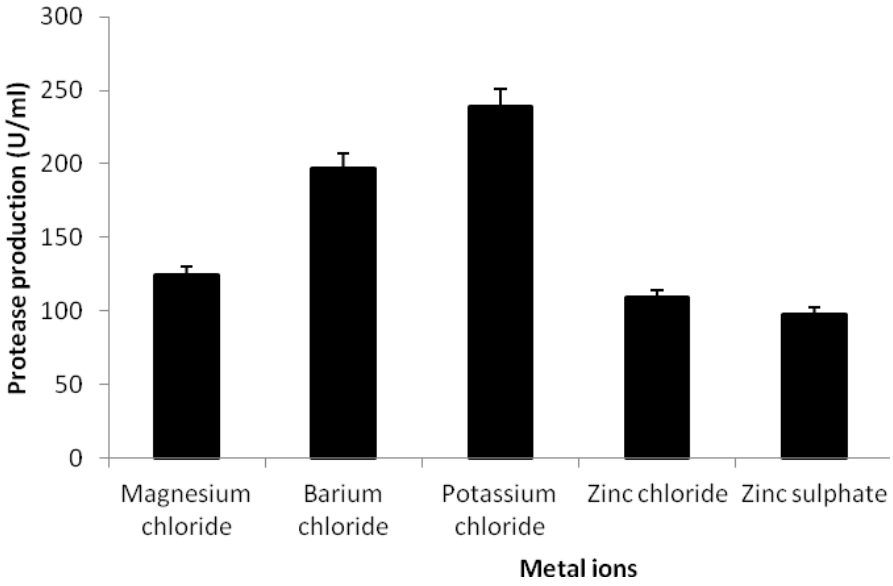

Figure 2b. Effect of different metal ions on protease production.

(Figure 2b) for higher protease production. Similar results have been reported in previous studies where fructose have been found as suitable substrates and inducers for the production of proteases by *Bacillus* sp. I-312 (Joo and Chang, 2005) and *Serratia proteamaculans* AP-CMST (Esakkiraj et al., 2011).

Using Plackett-Burman design, a total of seven variables were analyzed with regard to their effects on protease production (Table 1). The designed matrix was selected to screen the significant variables for protease production and the corresponding responses are shown in Table 2. Among the seven variables screened, four components such as anchovy waste, KH_2PO_4, NaCl and temperature were selected for further response surface methodology analysis based on their positive effect towards protease production (Table 2); fructose, KCl, and pH showed negative effect towards protease production. Even though the protein content of anchovy was higher than that of lipid that may be more suitable as an inducer for protease production, previous study by Esakkiraj et al. (2011) showed anchovy meal powder as a suitable inducer for the production of proteases by *S. proteamaculans* AP-CMST. Factors having a confidence level greater than 95% and significant 'p' value (less than 0.05) were considered to have a significant effect on the protease production. The optimal levels of the four selected variables (anchovy waste, K_2PHO_4, NaCl and temperature) and their interactions were then examined by a central composite design. Present findings are in line with several reports showing the enhancement of protease production in the presence of fish meal as nitrogen sources by *Bacillus mojavensis* A21 (Haddar et al., 2010; Esakkiraj et al., 2009, *Pseudomonas aeruginosa* MN7 (Triki-Ellouz et al., 2003).

Central composite design

The overall price of industrial protease production is very high due to higher cost of the substrates used. Therefore, development of novel process to increase the production of proteases using low cost substrate is important. Hence, microbial protease producing industries are always looking for new and cheaper methods to enhance protease yield of enzyme and reduce the market price (Haddar et al., 2010; Annamalai et al., 2013). In the present study, a total of 31 experiments with different combinations of the four selected variables were performed (Table 4). The central composite design, employed to determine the optimum levels of the four screened factors and six levels, including five replicates at the centre point, was used for fitting a second-order response surface. The variance (ANOVA) analysis is presented in Table 5. The mathematical model relating to the protease production with the independent process variables, X_1, X_2, X_3 and X_4 is given in the second-order polynomial equation.

$$Y= -15894.0 + 208.3X_1 + 185.2X_2 + 12.8X_3 + 630.8X_4 - 3.2X_1^2 - 64.5X_2^2 - 0.5X_3^2 - 7.3X_4^2 + 3.1X_1X_2 - 0.4X_1X_3 - 1.5X_1X_4 - 0.3X_2X_3 - + 3.6X_3X_4 + 0.9X_4X_6$$

Where, Y is the predicted protease yield, X_1 is anchovy wastes, X_2 is K_2PHO_4, X_3 is NaCl and X_4 is temperature. The regression coefficients and the analysis of the variance (ANOVA) indicate the high significance of this model. For a good statistical model, the high F-value and non-significant lack of fit indicate that the model is a good fit and all of the factors should be positive and close to each other. Also, significant P-values (0.000) suggested

Table 5. Analysis of variance (ANOVA) for the quadratic model.

Source	DF	Seq SS	Adj SS	Adj MS	*F* value	*P* value
Regression	14	628481	628481	44892	1839.33	0.000
Linear	4	47636	79000	19750	809.21	0.000
Square	4	542045	542045	135511	5552.27	0.000
Interaction	6	38801	38801	6467	264.96	0.000
Error	16	391	391	24.0		
Lack-of-Fit	10	297	297	30	1.90	0.223
Total	30	628872				

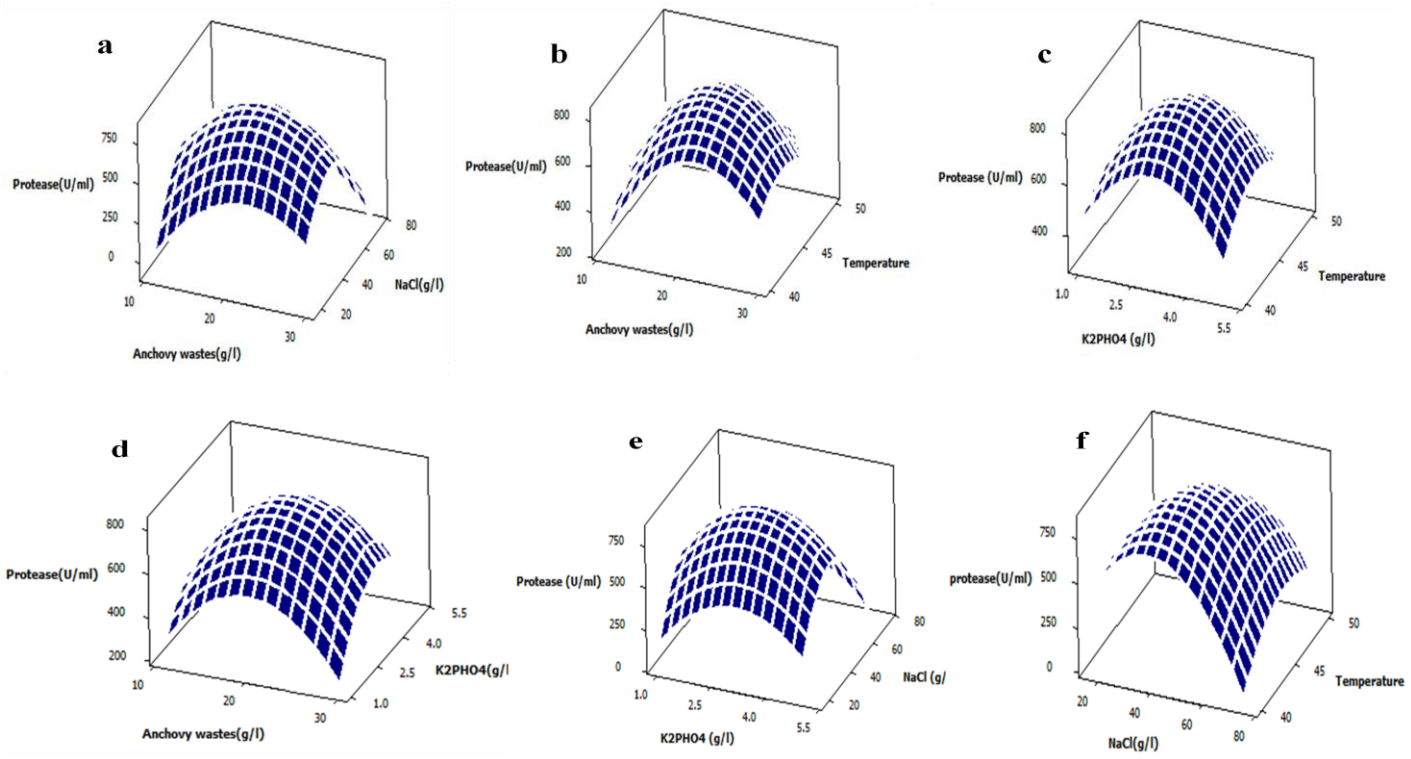

Figure 3. Three dimensional response surface plot for protease production showing the interactive effects of the anchovy wastes and NaCl **(a)**, anchovy wastes and temperature **(b)**, KH_2PO_4 and temperature **(c)**, anchovy wastes and K_2PHO_4 **(d)**, K_2PHO_4 and NaCl **(e)**, NaCl and temperature **(f)**.

that the obtained experimental data was a good fit with the model and the fit of the model was also checked by determination of coefficient (R^2) with R^2 (multiple correlation coefficient) of 99.94%. The predicted R^2 and the adjusted R^2 was about 99.71 and 99.88%, respectively. The regression coefficients of all linear, quadratic terms and two cross products are significant at 1% level. The three dimensional response surface plots were presented in Figure 3 (graphical representations of the regression equation) from which the protease production for different concentration of the variable could be predicted. The responses are plotted on the Z-axis against two variables while other variable was maintained at level zero.

The optimal values of anchovy waste, KH_2PO_4, NaCl and temperature were estimated as 20.50, 3.06, 42.91 (g/l) and 43.36°C, respectively, with a predicted protease production of 830.307 U/ml. The confirmation experiment was conducted for predicted optimum conditions and the protease production was about 842.102 U/ml. The protease production from the experiment was near about the value predicted by the software which reveals high accuracy of the model. Similarly, maximum protease productions were obtained for *Bacillus sp*. RKY3 (939 U/ml) (Reddy et al., 2008) and *B. mojavensis* A21 (1830.60 U/ml) (Haddar et al., 2010) under optimized

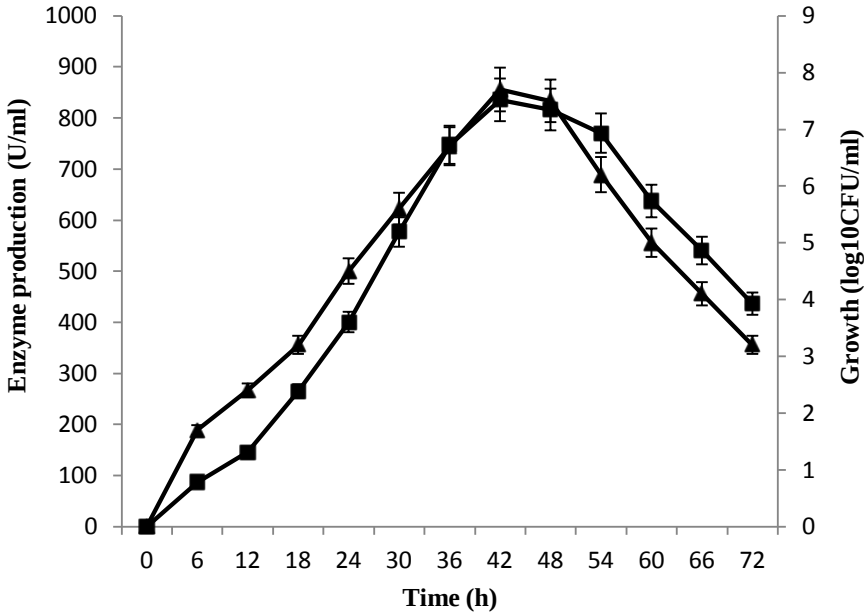

Figure 4. Kinetics of growth (CFU/ml) and alkaline protease production in optimized medium.

culture conditions by response surface methodology. However, protease production by *Bacillus* sp. GA CAS10 is much more than protease production by *B. subtilis* A26 (269.36 U/ml) (Agrebi et al., 2009), *Bacillus* sp. L21 (306.5 U/ml) (Tari et al., 2006), *Bacillus* sp. (410 U/ml) (Patel et al., 2005), *B. mojavensis* (558 U/ml) (Beg et al., 2003).

Time course of protease production and cell growth

The time course of protease production and the cell growth of *Bacillus* sp. GA CAS10 for the optimized (anchovy waste 20.50 g/l, KH_2PO_4 3.06 g/l, NaCl 42.91 g/l, temperature 43.36°C and pH 9) conditions is shown in Figure 4. These results show that the enzyme production in relation with incubation time and cell growth revealed that both bacterial cell growth and protease production reached maximum (856.29 U/ml) at 42 h and started to decrease gradually after 48 h. This optimization strategy led to the enhancement of protease from un-optimized conditions (267.33 U/ml) to optimized conditions (856.29 U/ml).

Conclusion

The microbial protease production by utilizing anchovy waste not only solves ecological troubles but also enhances the economic value of the wastes. The optimized medium established in this work might result in a significant reduction in the cost of medium constituents and would thus offer advantages for large-scale fermentation.

Conflict of Interests

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

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African Journal of Biotechnology

Full Length Research Paper

Evaluation of hypoglycaemic activity of ethanol extract of *Gongronema latifolium* (Asclepiadaceae) leaves

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The mechanism of anti-diabetic activity of *Gongronema latifolium* was evaluated. The ethanol extract of the leaves of *G. latifolium* were fractionated using solvents of increasing polarity, namely n-hexane, chloroform, ethylacetate and ethanol. Phytochemical screening of the dried fractions were carried and then acute toxicity tests on mice carried out. The induction of diabetes mellitus was achieved with 150 mg/kg b.w for mild diabetes and 300 mg/kg b.w for the severe condition. The effects of the crude ethanol extract (CEE) and its fractions on alloxan-induced hyperglycaemia were monitored. The result obtained reveals that crude ethanol extract significantly and dose-dependently reduced hyperglycaemia. The fractions of the ethanol extract equally reduced hyperglycaemia but the level of reduction was affected by the phytochemical content. This suggests that an intact pancreas is required for the hypoglycaemic action which is the mechanism of action of the sulphonylureas.

Key words: Anti-diabetic, *Gongronema latifolium*, hyperglycaemia, hypoglycaemia, phytochemical screening.

INTRODUCTION

Diabetes mellitus is a prototype of chronic disease and one of the world's leading causes of death, illness and reduced quality of life in both industrialised and industrialising nations of the world. It is therefore a major public health problem because of the direct and indirect cost of its treatment and the elevated morbidity and mortality of the disease (Matthews and Matthews, 2011). The British Medical Association (BMA) dictionary defines diabetes mellitus as a disorder caused by insufficient or absent production of the hormone insulin by the pancreas or because the tissues are resistant to the effects. Recently, DeFronzo et al. (2013) described the main factors involved in the diabetes pathophysiology, calling them the ominous octet: reduced peripheral insulin resistance, characterized by: 1) increased lipolysis, 2) reduction of glucose uptake by muscle, liver and adipose tissue, 3) beta cell dysfunction, with relative decrease in insulin production; 4) increased hepatic gluconeogenesis, 5) reduction of the incretin effect (optimization of insulin and glucagon glucose -dependent secretion, reduced gastric emptying, and induced satiety by GLP-1 - glucagon like peptide-1) present in the intestine, 6) increased renal glucose reabsorption, 7) hyperglucagonemia, and 8) neurotransmitter dysfunction. Either ways,

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Abbreviations: CEE, Crude ethanol extract; **nHE,** n-hexane extract; **CLE,** chloroform extract; **EAE,** ethylacetate extract; **REE,** residual ethanol extract.

hyperglycaemia generated in the blood triggers a whole chain of homeostatic disorders resulting in derengagement of metabolic processes of carbohydrates, proteins and fats (DeFronzo et al., 2013). Clinical conditions of the vascular system, kidney, eye, nerves and skin affect all ages and imposes huge economic burden on families.

Pharmacological intervention in the disease has a primary objective, which is targeted towards reducing hyperglycaemia and minimizing macrovascular and microvascular complications of the disease. According to United Kingdom Perspective Study (UKPDS), intensive glycaemic control will reduce risk of microvascular complications in patients with type 2 diabetes. Reduction of microvascular complications with intensive treatment of hyperglycemia were also observed in patients with type 1 diabetes (DCCT, 1993). This involves the use of insulin or oral hypoglycaemic agents. Hypoglycemic agents act on different pathways of diabetes and can restore the glycemia in different ways, such as increasing pancreatic insulin secretion, increasing glucose uptake by muscle and fat tissue, inhibiting hepatic glucose production, stimulating the incretin effect, reducing the renal or intestinal glucose uptake and directly replacing insulin (Inzucchi et al., 2012; Shlafer and Marieb, 1989). Based on the fact that hyperglycemia is caused by multiple factors, and none of the available drugs act on all metabolic disorders of diabetes, often becomes necessary to combined two or more drugs therapy to achieve glycemic targets. In their observation, UKPDS noted that despite the introduction of new oral hypoglycaemic agents for the management of diabetes mellitus, they have not yet been able to control the blood glucose level satisfactorily or reduce the complications of end organ anomalies associated with the disease. Thus, the discovery of new drugs or agents able to reduce glycemia and minimize complications of this disease is needed. Traditional antidiabetic plant treatment provides an object lesson in the functionality of foods (DCCT, 1993).

The active principles contained in medicinal herbs not only alleviate dysfunctions but also regulate life processes and prevent disease conditions. Phytochemical componenents such as flavonoids from *Pterospartum tridentum* (Middleton and Kandaswami, 1993), alkaloid, boldine from boldo *Peumus boldus molin* (Jang et al., 2000), and steroidal saponins from fenugreek *Trignolla foenum-graecum* (Sauvaire et al.,1996) have been shown to interfere with free radical production and cholesterol deposition as well as protect against diabetes. Others operate as insulin like substance (Li et al., 2005) or in pancreatic regeneration (Shanmugasundaram et al., 1990) in order to suppress blood glucose levels. These components are combined with complimentary components which give the plant as a whole a safety and efficiency much superior to that of its isolated pure active compound (Pamplona - Roger, 2001).

Gongronema latifolium (Utazi) is a Nigerian dietary vegetable attributed with many medicinal properties and listed among the medicinally important vegetables of South West Nigeria (Eleyimi, 2008). Both whole vegetable feeding (Eleyimi, 2008) and extracts of the leaf and stem have also been implicated in reduction of blood glucose levels (Akinnuga et al., 2011 and Eleyimi, 2008). However, the chemical components of the vegetable involved in reducing hyperglycaemia and their mechanism of blood glucose lowering have not been established (Akinnuga et al., 2011). This work is therefore aimed at identifying the phytochemical components associated with reduction of hyperglycaemia and the probable mechanism by which the blood glucose reduction is achieved.

MATERIALS AND METHODS

Plant materials

Fresh leaf samples of *G. latifolium* Benth Asclepiadaceae (Utazi in Igbo, aroeke in Yoruba) were purchased from vegetable markets in Nsukka, Nigeria. They were air dried under shade and pulverized. The sample was weighed and stored in airtight containers.

Extraction

Pulverized air-dried leaves (1 kg) of the *G. latifolium* were macerated in 5 L of 96% ethanol for 48 h and the Whatman No. 1 filtrate (CEE) was dried at 40°C and stored at -10°C for use. The CEE (75 g) was fractionated on silica using solvents of increasing polarity: n-hexane, chloroform, ethylacetate and ethanol. Each of the fractions was dried and stored for use. Phytochemical screening of crude ethanol extract and its fractions were carried out using standard biochemical methods of Harborne (1973).

Animals

Mice

Albino mice (21) weighing between (17 to 30 g) were purchased from Departments of Pharmacology and Toxicology animal house of University of Nigeria, Nsukka. They were housed in metal cages in the Department of Biochemistry animal house under standard conditions of 12 h light/dark cycles, fed with pelleted feed and water *ad libitum*.

Rats

About 80 inbred Wistar albino rats weighing (150 to 250 g) were purchased from the Faculty of Veterinary Medicine animal house of University of Nigeria, Nsukka. They were also housed in the Department of Biochemistry animal house in similar conditions as those of the mice. Ethical laws on the use of experimental animals were obeyed.

Acute toxicity test

The acute toxicity study of the crude ethanol extract was carried by Lorke (1983) method using mice. The first phase involved three

Table 1. Acute toxicity study of the crude extract.

Group	Dose (mg/kg)	Observation
1	10	No fatality
2	100	No fatality
3	1000	No fatality
4	1600	No fatality
5	2900	No fatality
6	5000	No fatality

Table 2. Phytochemical composition of the crude extract/sub-fractions and n-hexane fraction of *Gongronema latifolium.*

Group	Phytochemical component
Crude ethanol extract (CEE)	Resins, terpenoid, steroid, fats/oils, alkaloids, flavonoids, saponins, carbohydrates, reducing sugars, proteins, glycosides
n-hexane extract (nHE)	Resins, terpenoids, steroids, fats/oils
Chloroform extract (CLE)	Resins, terpenoids, steroids, fats/oils, alkaloids, flavonoids
Ethylacetate extract (EAE)	Resins, terpenoids steroids, fats/oils, flavonoids, glycosides
Residual ethanol extract	Resins, terpenoids, steroids, flavonoids, saponins, alkaloids, carbohydrates, glycosides, proteins, reducing sugars

groups of three mice each administered 10, 100 and 1000 (mg/kg b.w) solutions of the crude ethanol extract (CEE) intraperitonally. The animals were monitored for 24 h for fatalities and signs of toxicity. The second phase involved four groups of three mice each administered 1600, 2900 and 5000 (kg/b.w) or saline extract and observation for fatalities done.

Anti-diabetic study

The method of Abdel-Hassan et al. (2001) was used to determine the effect of crude ethanol extract on diabetes mellitus on wistar albino rats weighing between (150 to 250 g). After an overnight fast, diabetes mellitus was induced by intraperitoneal administration of aqueous solution of alloxan monohydrate (Sigma-Aldrich, USA) (150 mg/kg b.w) to five groups of five rats each with an uninduced sixth group. Diabetes was confirmed after 72 h of blood glucose level above 300 mg/dl from blood collected from the tail (Al-Hader et al., 1994). Varied doses of the crude ethanol (50, 100 and 300 mg/kg b.w.) fixed dose of the standard drug (100 mg/kg) and the control were administered orally to the appropriate groups and blood glucose levels were monitored at intervals of 0, 1, 3, 6, 12 and 24 h. The percentage reduction in blood glucose level was computed for each dose of extract and the standard drug.

A second set of six groups of five rats each and a control (uninduced) were also treated with the same dose of the alloxan (150 mg/kg b.w). This time a fixed dose of the four fractions of the ethanol extract, the control and standard drug were administered orally according to the procedure described above.

A third set of five groups of five rats each and a sixth group (uninduced) were administered with higher dose of alloxan (300 mg/kg b.w) according to procedure described above. Fixed doses of crude and four fractions (300 mg/kg b.w) were administered orally and blood glucose levels monitored at those hours.

Statistical analysis

Data from the study was analysed by SPSS version18 using one way analysis of variance and subjected to Fischer LSD post HOC. Results were expressed as mean ± SEM. Differences between means were considered significant at $p<0.05$.

RESULTS

The result in Table 1 reveals that none of the mice died even at highest doses of the extract (2900 and 5000 mg/kg). Therefore, the extract is safe and non-toxic for use. These fractions were known to exhibit pharmacologically active phytochemicals namely flavonoids, saponins, alkaloids, glycosides, steroids, terpenoids, fats, oils and resins to name a few. The fractions of the crude varied in their composition of phytochemicals as shown in Table 2.

The result from Table 3 shows that the glibenclamide significantly ($p < 0.05$) reduced mean blood glucose level (85%). Similarly and dose-dependently, the ethanol extract inhibited blood glucose level by 75.3, 80.4 and 83.2% at 50, 100 and 300 mg/kg doses, respectively. However reduction in blood glucose level was more consistent over the period of time with 100 mg/kg extract than the other test doses. In addition, the standard drug gave the highest blood glucose level inhibition (85.2) than the highest dose of extract (83.2).

Table 3. Effect of the crude ethanol extract of *Gongronema latifolium* leaves on 150 mg/kg b.w. alloxan-induced diabetes mellitus.

Treatment group	Dose	Mean blood glucose concentration (mg/100 ml)/Time (h)						
		0	1	3	6	12	24	% Reduction after 24 h
Normoglycaemic	5 ml	124.25 ± 5.5[a]	114.50 ± 8.0[a]	88.25 ± 20.8[a]	83.50 ± 6.8[a]	77.00 ± 3.7[a]	59.25 ± 4.5[a]	52.30
Diabetic (CEE)-treated	50 mg	365.00 ± 23.4	344.25 ± 551.1	309.50 ± 45.6	261.75 ± 82.6	245.00 ± 88.9	90.00 ± 10.1	75.30
Diabetic (CEE)-treated	100 mg	435.50 ± 54.3	406.00 ± 74.3	330.50 ± 42.3	315.00 ± 56.9	148.25 ± 22.1	85.25 ± 6.2[a]	80.40
Diabetic (CEE)-treated	300 mg	427.00 ± 62.2	455.50 ± 53.50	434.75 ± 58.6	470.50 ± 54.9	434.75 ± 70.4	71.75 ± 9.9[a]	83.20
Diabetic-Untreated: Tween/solution	5 ml	356.25 ± 23.67	429.50 ± 21.9	363.50 ± 27.1	384.00 ± 19.0	341.25 ± 22.5	270.00 ± 9.2	24.2
Diabetic- treated glbenclamide	100 mg	460.75 ± 23.6	257.50 ± 22.4[a]	200.25 ± 10.6[x]	156.50[x] ± 24.5	118.25 ± 28.6[x]	68.25± 9.2[a]	85.2

Values presented as mean ± SEM, [x] $P<0.05$ [a] $P<0.001$ against negative control.

Table 4. Effect of CEE and fractions on blood glucose level at 300 mg/kg alloxan-induced diabetes in rats.

Treatment group	Dose (mg/kg)	Mean blood glucose concentration (mg/100 ml)/Time (h)						
		0	1	3	6	12	24	% Reduction
Normoglycaemic	5 ml	124.25 ± 5.5	114.50 ± 8.9	88.25 ± 20.8	83.50 ± 6.8	77.00 ± 3.7	59.25 ± 4.5	52.3
Diabetic CEE- treated	300	513.00 ± 40.0	419.0 ± 49.0	412.00 ± 36	433.0 ± 22.0	401.00 ± 25.0	418.00 ± 26.00	18.52
Diabetic n-HE- treated	300	519.00 ± 12.0	494.00 ± 23.0	523.0 ± 69.0	523.0 ± 53	515.0 ± 46	495.00± 39.0	4.6
Diabetic CLE- treated	300	390.00 ± 40.0	347.50 ± 35.0	371.00 ± 36.0	424.0 ± 41.0	370.0 ± 28.0	432.00 ± 84.0	5.13
Diabetic EAE- treated	300	468.00 ± 52	517.0 ± 32.0	599.0 ± 0.7	600.0 ± 0.0	520.0 ± 32.0	562.00 ± 23.0	6.30
Diabetic REE- treated	300	464.00 ± 33	491.00 ± 65.0	524.0 ± 38.0	536.0 ± 25.0	545.0 ± 28	502.00 ± 8.0	7.89
Diabetic- untreated	300	593.800 ± 55.0	592.0 ± 8.0	600.0 ± 0.0	600.0 ± 5.0	592.0 ± 8.0	556.17 ± 47	6.33
Diabetic treated glibenclamide	100	374.00 ± 33.0	297.0 ± 6.0	218.0 ± 24.0	152.0 ± 19.0	113.0 ± 9.6	77.0 ± 2.0	79.4

No significant difference of means between treated groups.

Effect of CEE and its fractions on blood glucose level

The mean blood glucose level is recorded in Table 4. The result shows that the extracts and glibenclamide significantly ($p<0.05$) reduced blood glucose with respect to the untreated. The percentage blood glucose levels of REE, EAE, CLE and n-HE were 77.9, 76.4, 72.1 and 56.1%, respectively. However, glibenclamide gave higher reduction of blood glucose level (83.47%).

Effect of crude and its fractions at severe induction of diabetes

The mean blood glucose level of crude ethanol extract and its fractions at severe induction of diabetes is shown in Table 4. There was no significant reduction of blood glucose level by the extracts but glibenclamide still showed reduction in blood glucose level.

DISCUSSION

The results from this work show that *G. latifolium*

leaf extract contained important phytochemicals such as terpenoids, steroids, flavonoid, alkaloids and saponins in the other and glycosides associated with anti-diabetic activities. Many studies have implicated these fiber, alkaloids, flavonoids such as quercitin, kaempferol and caffeoyl glucoside as well as saponins and glycosides have all been demonstrated to inhibit hyperglycaemia in animal models (Shimizu et al., 2001 and Abdel- Hassan et al., 2001). Therefore, it can be adduced that the presence of such phytochemicals in the *G. latifolium* leaf extract might account for its hypoglycaemic activity.

The crude extract (CEE) in this study evoked a significant ($P<0.05$) and dose-dependent reduction in blood glucose level.

The effect of the phytochemicals in blood glucose reduction is demonstrated in the result obtained by the fractions of the crude extract. It was also observed that the extracts were inadequate to bring down the blood glucose level when alloxan-induction was severe at 300 mg/kg b.w (Table 4). Alloxan attacks pancreas depleting the beta cells responsible for releasing insulin, the hormone responsible for glucose metabolism (Shimizu et al., 2001). The ineffectiveness of the fractions, therefore, suggests that the extracts might have induced reduction in hyperglycaemia by stimulating the pancreatic beta cells to release insulin, and so in the case of severe damage to the pancreas their potency was lost. This mechanism corresponds to the function of sulphonylureas as oral hypoglycaemic agent (Shlafer and Marieb, 1989). Another member of the Asclepiadaceae family as *G. latifolium*, namely, *Gymnema sylvestre* with potent hypoglycaemic activity has been reported to operate by the same mechanism (Shanmugansundaram et al., 1990). These reporters also implicated saponins in the hypoglycaemic activity of the medicinal plant. In a previous work Shlafer and Marieb (1989), reported that components of the vegetable inhibited alpha glucosidase activity, which is another mode of action of oral hypoglycaemic drugs.

The outcome of this study validates the use of *G. latifolium* as a medicinal plant therapy for diabetes mellitus. The components of the vegetable require an intact pancreas for maximum activity, possibly to increase insulin production, which is the mode of operation of the sulphonylureas.

Conflict of Interests

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

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Anti-ulcer and gastric anti-secretory activities of seed extract of *Buchholzia coriacea* in Wistar Albino rats

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Ethanol extract of *Buchholzia coriacea* seed was evaluated for anti-ulcer as well as anti-secretory activity in rats because of its use in Nigerian folk medicine as an anti-ulcer agent. Standard pharmacological methods were used to carry out phytochemical analysis of the plant. Quantitative phytochemical analysis of the ethanol extract of *B. coriacea* revealed the presence of alkaloids (101.88 ± 0.11 mg/100 g), flavonoids (46.88 ± 2.21 mg/100 g), tannins (0.16 ± 0.02 mg/100 g), oxalate (0.15 ± 0.01 mg/100 g) and terpenes (23.0 ± 0.30 µg/100 g). The extract at 200 and 400 mg/kg body weight, significantly (P<0.05) and dose-dependently suppressed the ulcerogenic effect induced by indomethacin in rat gastric mucosa relative to the controls. Similarly, the extract significantly (P<0.05) decreased histamine-mediated gastric acid secretion and also blocked histamine-induced contractile responses in isolated guinea-pig ileum in a similar fashion as the standard anti-histamine drug, chlorpheniramine. The extract had comparable ulcer protective potency with cimetidine, which is a standard drug used in the management of ulcer. The mechanism of the extract's efficacy to protect the animals against indomethacin-induced ulcer may be diverse in nature (due to the presence of a number of bioactive constituents) but suppression of mediator effect of histamine is likely to play a predominant role in the observed activity.

Key words: *Buchholzia coriacea*, anti-ulcer, cimetidine, anti-secretory and indomethacin.

INTRODUCTION

Gastric ulcer is an inflammed break in the lining of the stomach caused by increased acid production or damage to the mucus lining of the stomach (Goel et al., 1985). In most conditions, the event of gastric ulcer involves an imbalance between aggressive factors (gastric acid, pepsin, and *Helicobacter pylori*) and protective factors (mucin, prostaglandin, bicarbonate, nitric oxide, and growth factors) (Hoogenwerf and Passrichas, 2001). The conventional anti-ulcer drugs such as proton pump inhibitors, histamine receptor antagonists, prostaglandin analogues and drugs affecting mucosal barrier are currently in use (Dharmani and Palit, 2006). While the use of these drugs may be effective, they are usually expensive and sometimes associated with relapse and adverse effects (Dharmani and Palit, 2006). This has led to renewed interest in the search for new anti-ulcer drugs from natural sources. Many plants have been reported to possess anti-ulcer activities. Such plants include

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Hemidesmus indicus (Anoop and Jegadeesanj, 2003), *Asparagus racemosus* (Sairam et al*.,* 2003), *Desmodium gangeticum* (Dharmani et al*.,* 2005), *Combretum racemosum* (Okwuosa et al*.*, 2006), *Corindrum sativum* (Al-Mofleh et al., 2006), *Sida acuta* (Landeswari et al*.*, 2010) and *Cissus quadrangularis* (Enechi et al., 2013a).

Buchholzia coriacea is a perennial shrub which is widely distributed in the tropical regions of West Africa. The description of the plant morphology has been documented (Hutchinson and Dalziel, 1954). In Nigeria, the seed is commonly referred to as "wonderful kola". It is widely used for traditional herbal treatment of inflammation, ulcer, bronchitis, mild fever, headache, tonsillitis and catarrh (Moser et al*.*, 2007). It is also used to treat a number of bacterial infections (Moser et al*.*, 2007). For use, the seeds are cut into bits and soaked in local gin or clean water for some days. The mixture is then filtered and the filtrate is taken orally using measurable crucible, depending on the severity of the illness. The bark is dried and ground to form powder which is snuffed through the nostril for the relief of headache, migraine and nasal congestion.

The bark sap is also applied for chest pain, bronchitis and kidney pains. In Gabon, the leaves are used for the treatment of boils. The fruits are used for the treatment of fever and as antihelminthic (Walker, 1953).

Studies have shown that the seeds have antibacterial activity (Ezekiel and Onyeoziri, 2009) as well as antihelminthic activity (Ajaiyeoba et al., 2001). The anti-inflammatory, analgesic and antipyretic activities (Enechi et al., 2009) as well as the hypolipidemic potentials (Enechi et al*.,* 2013b) of the plant seed have also been reported. The pulverized seed of the plant has been demonstrated to be a good source of antioxidant vitamins (Enechi, 2011). Also, the plant has been shown to exhibit significant ($P<0.05$) free radical scavenging and anti-lipid peroxidation activities (Enechi, 2011). Although *Buchholzia* seed is used as an anti-ulcer agent in Nigerian folk medicine, there is no empirical evidence of the anti-ulcer activity. Thus, the present study was undertaken to evaluate the gastro- protective activity of the ethanol extract of *B. coriacea* against indomethacin-induced gastric ulceration.

MATERIALS AND METHODS

Pant material

Buchholzia coriacea seeds were collected from Nsukka, Enugu State, Nigeria and then identified by Mr. A. Ozioko of Bioresources Devleopment and Conservation Programme Center, Nsukka, Nigeria. Voucher specimens were retained in the Herbarium, Department of Plant Science and Biotechnology, University of Nigeria Nsukka, Nigeria.

Animals

The rats used in this study were obtained from the Animal House of the Faculty of Biological Science, University of Nigeria, Nsukka. Albino rats (Wistar strain) weighing 150 -200 g of either sex were used. The rats were housed in metal cages for at least one week in the animal room of Biochemistry Department, University of Nigeria, Nsukka, prior to testing. They were maintained under standard environmental conditions and 12 h light and dark cycles. The animals were allowed free access to standard pellets and clean water *ad libitum.* The laboratory animals were used in accordance with laboratory practice regulation and the principle of humane laboratory animal care as documented by Zimmermann (1983).

Chemicals

The chemicals used in this work were of analytical grade and included: chloroform (Riedel De Haen (RDH), Germany); ethanol (absolute) (British Drug Houses, Dorset, U.K); sodium chloride, histamine and chlorpheniramine (May and Baker, Dagenham, England); cimetidine (Sigma Chemical Company, St. Louis, U.S.A); and indomethacin (Emzor pharmaceuticals, Nigeria).

Extraction

Fresh seeds of *B. coriacea* were air dried at room temperature for two weeks and milled to a coarse powder with a Crestow milling machine. The pulverized seed was subjected to cold maceration by methods corresponding to those practiced by Nigerian traditional doctors (Safowora, 1993). One kilogram quantity of the pulverized seeds was macerated in 5 volume (w/v) 70% ethanol for 24 h with two changes of solvent. The filtrate (extract) was concentrated using a rotary evaporator. The residue was stored in a refrigerator at 4°C until used.

Phytochemical analysis

Phytochemical analysis was done using standard methods as described by Harborne (1973) and Onwuka (2004)

Indomethacin induction of ulcer

The effect of *B. coriacea* seed extract on ulcer formation was evaluated as described by Urushidiani et al*.* (1979). Prior to the test, the rats were starved of food for 24 h but they had free access to water. Twenty (20) rats were employed and grouped into four (A - D) of five rats each. Group A received 5ml/kg of b.w of normal saline p.o and served as negative control. Group B received cimetidine (100 mg/kg b.w p.o), Group C received 200 mg/kg b.w. of the extract, while Group D received 400 mg/kg b.w.of the extract (p.o). 30 min later, ulcer was induced in each rat by the oral administration of indomethacin (40 mg/kg) suspended in normal saline (5 ml/kg) to the different groups of animals. After 8 h, the animals were sacrificed and the stomachs quickly dissected out. The stomach of each animal was opened along the line of greater curvature, rinsed under a stream of water and pinned flat on a cork board. The ulcers were viewed with the aid of a magnifying lens (X10) and each given a severity rating (Main and Whittle, 1975) as follows: <1 mm=1, >1 mm<2 mm=2 and >2 mm<3 mm=3. The overall total scores divided by 10 was designated the ulcer index (UI) for that stomach. The percentage ulcer inhibition was calculated according to the method of Suzuki et al. (1976) using the following formula:

Percentage Ulcer inhibition (% UI) = (1- Ut/Uc) × 100

Where, Ut represents the ulcer index of the treated group and Uc represents the ulcer index of the control group.

Determination of the effect of the extract on histamine-induced gastric acid output

The effect of *B. coriacea* seed extract on gastric acid secretion was studied on lumen perfused rat isolated stomachs by modifications of the method of Brunce and Parson (1978) as described by Nwodo et al. (2008). Five albino rats were fasted for 18 h after which they were killed by chloroform inhalation in an air-tight plastic container and the stomachs dissected out. The stomachs were quickly washed with the modified Krebs' solution (deprived of sodium bicarbonate) at room temperature. A stomach strip was placed into series of beaker labeled A-E, each containing 10 ml Krebs solution. The pH of beaker A, containing only stomach strips and Krebs' solution was determined after 5 min using a pH meter. To beaker B, a sub-maximal concentration of histamine (H) (20 µg/ml) was added and the pH of the solution was determined and recorded after 5 min. To beakers C, D and E; 0.2 mg/ml, 0.5 mg/ml and 1.0 mg/ml of the extract (E) were added respectively. After 2 min, histamine (20 µg/ml) was added to each of them and the pH values of the solutions were determined after 5 min. Acid outputs were obtained by calculating the mean pH values for 3 determinations in the respective incubates.

Effect of the extract on histamine-induced contraction of isolated guinea pig ilium

Evaluation of the effect of the extract on isolated guinea pig ileal contraction induced by histamine was carried out according to the method described by Capaso et al. (1988). Freshly isolated segments (2 cm long) of guinea pig ileum were suspended in a 10 ml organ bath containing Tyrode's solution constantly aerated and maintained at 37°C. A period of 30 min was allowed for tissue equilibration with bathing liquid. Responses of the tissue to varying doses of histamine and *B. coriacea* injection were recorded on Kymographic paper attached to a rotating drum. The contact time of each drug was 15 s with a 5 min cycle time.

Statistical analysis

The data obtained in this study were evaluated using the one-way analysis of variance (ANOVA) test between two mean groups, control and test groups, followed by Student's t-test. Significant levels were at $p<0.05$. Values were expressed as means ± standard deviation (SD)

RESULTS

Quantitative phytochemical composition of *Buchholzia coriacea* seed extract

The result of quantitative phytochemical analysis revealed the presence of high concentrations of alkaloids and flavonoids, and moderate concentrations of tannins and oxalate, while terpenes were present in low concentrations.

Effect of the extract on histamine-induced gastric acid output (pH) in rat stomach

The data presented in Table 2 shows that histamine caused an efflux of proton (H) as evidenced by the decrease in pH from 5.135 ± 0.007 to 4.705 ± 0.007. The extract at concentrations of 0.5 and 1.0 mg/ml induced significant ($p<0.05$) increase in pH from 4.705 ± 0.007 to 5.185 ± 0.049 and 5.745 ± 0.035, respectively, pointing to the ability of the extract to antagonize the acid efflux which leads to acid secretion and ulceration.

Effect of the extract on indomethacin-induced ulcer formation

Table 3 shows the effect of the extract on indomethacin-induced gastric ulceration. The ethanol extract of *B. coriacea* seed suppressed the ulceration induced by indomethacin as evidenced by the significant ($p<0.05$) dose-dependent decrease in ulcer index in the animals that were concurrently administered both the extract and indomethacin. Also cimetidine (a standard anti-ulcer drug) inhibited the ulcerogenic effect of indomethacin in a similar fashion to the inhibition by the extract.

Effect of the extract on histamine-induced contraction of isolated guinea-pig ileum

Figure 1a represents the effect of *B. coriacea* seed extract on histamine-induced contraction. When histamine was injected into the organ bath containing the isolated-guinea pig ileum preparation, there was marked contraction of the tissue. On concurrent administration of varying doses of the extract and histamine, the extract caused a reduction in the amplitude of the contractile response and this was dose-dependent. On its own the extract did not contract the tissue when administered via the bath.

Figure 1b represents the effect of a standard histamine antagonist, chlorpheniramine on histamine-induced contraction of isolated guinea- pig ileum. The figure shows that in the presence of the anti-histamine drug, the histamine-induced contractile response was blocked. The figure also shows that chlorpheniramine blocked the histamine-induced contraction of isolated guinea-pig ileum in a fashion similar to the blockade by the extract.

DISCUSSION

Extract of *B. coriacea* is used in folk medicine for the treatment of ulcers, bacterial and fungal infections, as well as malaria and inflammation (Awaad et al., 2008). In the present investigation, the ethanol extract of the plant showed potency as anti-ulcer agent against indomethacin- induced ulceration in rats. The evidence that indomethacin produced ulcers in all rats that received no further treatment (Table 3) is in consonance with the fact that indomethacin, like many anti-inflammatories, induces ulcers. Like cimetidine, the extract inhibited the ulcerogenic effect of indomethacin.

Table 1. Phytochemical composition of *Buchholzia coriacea* seed extract.

Phytochemical	Concentration (mg/100 g)
Alkaloids	101.88 ± 3.61
Flavonoids	46.88 ± 2.21
Tannins	0.16 ± 0.02
Oxalate	0.15 ± 0.01
Terpenes	$0.023 \pm 0.03 \times 10^{-3}$

Each value is the mean of 3 determinations ± S.D.

Table 2. Extract antagonism of histamine-induced gastric acid output by rat stomach strips

Treatment	pH
Krebs' solution + SS	5.135 ± 0.007
Krebs' solution + SS + HA	4.705 ± 0.007
Krebs' solution + SS + HA + Extract (0.2 mg/ml)	4.915 ± 0.092
Krebs' solution + SS + HA + Extract (0.5 mg/ml)	*5.185 ± 0.049
Krebs' solution +SS + HA + Extract (1.0 mg/ml)	*5.745 ± 0.035

SS = Stomach strips; HA = histamine (20 µg/ml). Each value represents mean ± S.D. of 3 determinations. *Significantly different at $p < 0.05$ relative to the control.

Table 3. Effect of the extract on indomethacin - induced ulcer in rat stomach

Group	Treatment	Mean ulcer index	Inhibition (%)
1	Normal saline (5 ml/kg body weight)	0.00 ± 0.00	-
2	Normal saline (5 ml/kg body weight) + extract (200 mg/kg body weight)	0.00 ± 0.00	-
3	Indomethacin (30 mg/kg body weight) + extract (200 mg/kg body weight)	*10.00 ± 0.12	32.20
4	Indomethacin (30 mg/kg body weight) + extract (400 mg/kg body weight)	*7.75 ± 0.50	47.46
5	Indomethacin (30 mg/kg body weight) + cimetidine (100 mg/kg body weight).	*5.90 ± 0.55	60.00
6	Indomethacin (30 mg/kg body weight)	14.75 ± 0.93	-

Each value represents mean ± S.D. (n=5). *Significantly different at $p < 0.05$ relative to the control.

This activity was dose-dependent and reveals that the extract has anti-ulcer effect.

Ulcer index is an established indication of ulceration in experimental animals. When rats were treated with only normal saline and also *B. coriacea* extract in normal saline, the ulcer index was zero revealing that neither the physiological saline nor the extract induced ulcers. The data presented in the Table 3 also show that indomethacin, a standard anti-inflammatory drug, induced a large scale of ulcer index. This is in consanant with well-established fact that the administration of many inflammatories on empty stomach produced ulcers.

There are multiple aetiologic factors in ulcer pathogenesis and the ability of the extract to protect against indomethacin- induced ulceration indicate its ability to inhibit one or more multiple inciting stimuli in ulcerogenesis (Awaad et al., 2008). Phytochemical analysis revealed the presence of bioactive constituents such as alkaloids, flavonoids, tannins and saponins with alkaloids and flavonoids occurring in high concentrations (Table 1). Hydrolysable tannins contain glucose moiety and have been used internally as astringent and as heavy metals antidote (John and Onabanjo, 1990).

Tannins being astringent may precipitate microproteins on the site of ulcer thereby forming an impervious protective pellicle on the lining to resist the attack of proteolytic enzymes (John and Onabanjo, 1990). This could be likened to the effect of drug such as sucralfate which act by providing a cytoprotective defense against acid peptic digestion. This is consistent with a previous report that partly attributed the anti-ulcer activity of crude extract of *Microgramma squamulosa* to the astringent action of tannins (Suffredini et al., 1999). Previous *in vitro* assessment of the antioxidant status of the ethanol extract of *Buchholzia coriacea* showed that the extract produced significant ($p<0.05$) antioxidant effect by

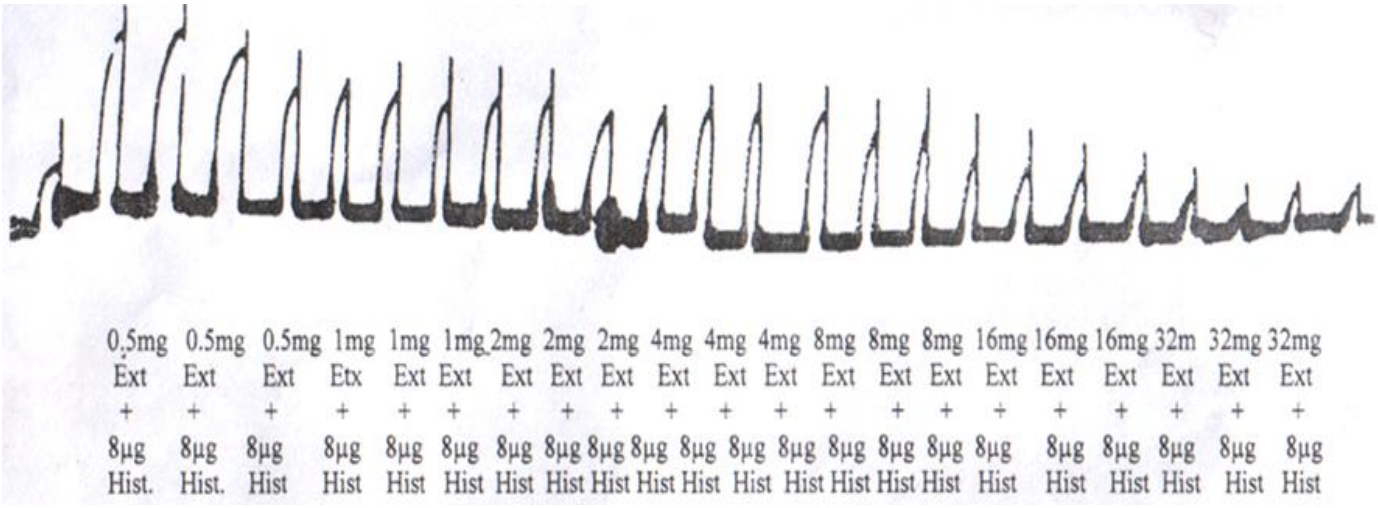

Figure 1a. Dose-dependent (0.5 to 32 mg/ml) blockade of *Buchholzia coriacea* seed extract on histamine-induced (8 µg) contraction of isolated guinea-pig ileum.

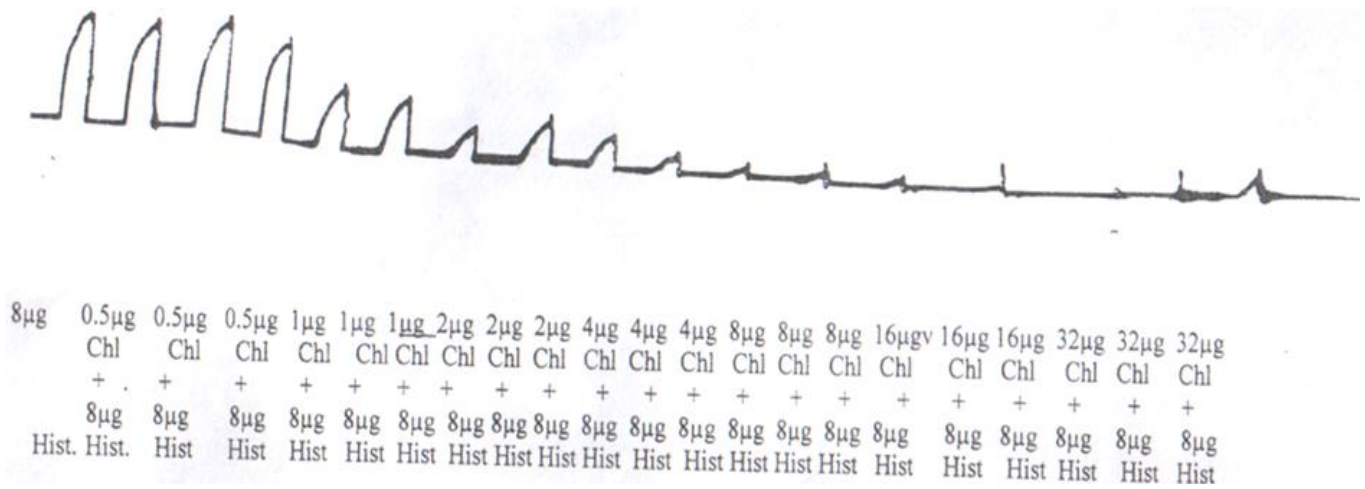

Figure 1b. Dose-dependent (0.5 to 32 mg/ml) blockade of chlorpheniramine (reference anti-histamine drug) on histamine-induced (8 µg) contraction of isolated guinea-pig ileum.

exerting significant ($p<0.05$) nitric oxide free radical scavenging activity (Enechi, 2011). The extract also significantly inhibited ferrous sulphate and CCl_4-induced lipid peroxidation in rat liver homogenate (Enechi, 2011). Anti-oxidants play a protective role against cellular damage by scavenging free radicals (Szabo, 1989). The extract may therefore exert a cytoprotective effect against ulcer formation by inhibiting free radical-mediated cell damage. The anti-ulcer activity of the extract may be due to flavonoids, which are abundant in the extract and have been shown in previous studies to possess anti-ulcerogenic and anti-ulcer activities (Tamotsu et al., 1978). Flavonoids, which are in relative abundance in this plant (46.88 ± 2.21 mg/100 g) are associated with free radical scavenging activity (Musonda and Clipman, 1998) and have been shown to protect various cell types from oxidative stress-mediated cell injury (Nakayama et al., 1993; Sasaki et al., 2002) and the flavonoids may play some roles in scavenging of free radicals in ulcerogenesis. Inhibition of prostaglandin synthesis is considered as the biochemical mechanism for the action of anti-inflammatory drugs (Vane, 1971). Ulcer induction may thus be consonant with inhibition of prostaglandin synthesis. Prostaglandins regulate many physiological processes including secretion of mucus that protect the gastric mucosa from acid and proteolytic enzymes in the stomach. Prostaglandins also inhibit gastric acid secretion. Histamine (H_2) receptor activity stimulates adenylate cyclase system and in turn causes increases in calcium ion concentrations (Nwodo et al., 2003), which ultimately

leads to activation of proton pump and consequently leads to hyperacidity and ulcer (AL-Mofleh et al., 2006; Awaad et al., 2008). It is likely that protection by the extract against indomethacin-induced gastric ulceration is achieved by the suppression of acid secretion. This could be likened to the effect of anti-ulcer drugs such as proton pump inhibitors which act by providing cytoprotective defense against gastric acid secretion (Ganong, 1995). This is corroborated by the findings in the present investigation (Table 2) that the extract significantly, and in a dose-dependent manner, suppressed the histamine-induced proton efflux in the stomach, which leads to acid secretion and ulceration Thus, the mechanism of ulceration by *B. coriacea* may be by competitively binding to H_2 receptors which histamine needs to bind in order to cause H^+ efflux in the stomach. Similarly, the extract blocked histamine-induced contractile responses in a similar fashion as the standard anti-histamine drug, chlorpheniramine. The antagonism of histamine-induced contractile response by the extract seemed to suggest that the extract may possess inhibitory activity at histamine receptor sites. The anti-histamine effect of the extract could be due to one or several phytochemicals present in the plant seed. Flavonoids which occur in relative abundance in this plant have been demonstrated to antagonize the effects of histamine which is a major mediator in ulcerogenesis (Sharma et al., 1996; Macander, 1986; Capasso et al., 1988; Middleton et al., 2000).

Conclusion

The results show that the ethanol extract of *B. coriacea* seed possesses anti-ulcer activity. The ability of the extract to suppress histamine-induced gastric acid secretion as well as to antagonize histamine-induced contractile responses indicates that suppression of mediator effect of histamine might be a likely mechanism through which the extract exerts its anti-ulcer activity. The anti-ulcer effect of the extract could be due to one or several phytochemicals that have been shown to be present in the plant seed. The results also provide empirical evidence for the use of the plant seed extract for the treatment of peptic ulcer in Nigerian folk medicine. The plant can be further harnessed as a potential source of novel anti-ulcer (bioactive) compounds.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Selection of inoculum size and *Saccharomyces cerevisiae* strain for ethanol production in simultaneous saccharification and fermentation (SSF) of sugar cane bagasse

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The aim of this work was to select an inoculum concentration and a *Saccharomyces cerevisiae* strain for ethanol production in the simultaneous saccharification and fermentation (SSF) of sugar cane bagasse. Three concentrations of inoculum (0.4, 4.0 and 8.0 g/L) and two strains of *S. cerevisiae* (UFPEDA 1238 and UFPEDA 1334) were used to ferment a culture medium containing glucose as the carbon source (100 g/L). Ethanol production was lower with 0.4 g/L inoculum, independent of the strain used. Experiments with 4.0 and 8.0 g/L inoculum showed no growth and higher ethanol production. Maximum ethanol concentration was obtained with UFPEDA 1238 and 8.0 g/L inoculum concentration. These conditions were selected for ethanol production from sugar cane bagasse in SSF. Maximum ethanol concentration was attained with SSF (28 g/L), enzymatic convertibility of cellulose (76%) and volumetric productivity (0.93 g/L h).

Key words: Ethanol, *Saccharomyces cerevisiae*, inoculum, efficiency.

INTRODUCTION

The amount of inoculum used is one of the most important factors that influences industrial fermentation, as well as lag phase duration, specific growth rate, biomass yield and the quality of the final product. Although the effect of a large quantity of inoculum on reducing the duration of the lag phase is well known, the relationship between product yield and inoculum has not been widely reported (Chen and Hashimoto, 1996).

Yeast inoculum size has a significant effect for ethanol production (Turhan et al., 2010). Gibbons and Westby (1986) reported that a 5% inoculum (v/v) resulted in rapid yeast and ethanol production. Higher inoculum showed no advantages. Tahir et al. (2010) using a different inoculum at 1-5% (v/v) observed that the amount of ethanol produced gradually increased with the increase in the inoculum. However, it was found that maximum ethanol production was achieved at 3% (v/v) inoculum. Results of Izmirlioglu and Demirci (2012) showed that 3%

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(v/v) inoculum was optimum for maximum ethanol concentration and production rate. Statistical analysis has revealed a significant effect of varying inoculum on ethanol production from sugarcane bagasse (Asgher et al., 2013).

Despite the reports on the increase in ethanol production by given inoculum, the initial biomass concentration was unclear. Therefore the aim of this work was to select an inoculum concentration (0.4, 4.0 and 8.0 g/L initial biomass concentration) and a *Saccharomyces cerevisiae* strain for ethanol production in the simultaneous saccharification and fermentation (SSF) of sugar cane bagasse.

MATERIALS AND METHODS

Microorganism and fermentation

Two industrial strains (UFPEDA 1238 and UFPEDA 1324) of *S. cerevisiae*, provided by the Culture Collection of the Department of Antibiotics of the Federal University of Pernambuco, Brazil, were used. These strains were maintained in a solid medium containing (in g/L) glucose (20), yeast extract (4), peptone (3) and agar (15), at pH 7.0. Inoculum was prepared by transferring cells of *S. cerevisiae* into a 500 mL flask containing 100 mL of the culture medium (20 g/L glucose, 3 g/L peptone, 4 g/L yeast extract; pH 7.0), and incubating this at 30°C for 12 h. Cells were harvested by filtration (0.45 µm filter), suspended in 10 mL sterilized water and used to inoculate the fermentation medium (Santos et al., 2012): 100 g/L glucose, 4 g/L yeast extract, 2 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4 and 0.75 g/L $MgSO_4.7H_2O$. The pH was adjusted to 5.5.

Ethanol production was carried out at 34°C without agitation and in duplicate, with 0.4 (A), 4 (B) and 8 (C) g/L of the inoculum in 250 mL flasks with a working volume of 100 mL. Samples were withdrawn after 12 h, filtered (0.45 µm filter), and the cell free supernatant was used to determine the glucose and ethanol by high performance liquid chromatography.

Steam-pretreated sugarcane bagasse and delignification

Sugarcane bagasse, pretreated by steam explosion at 200°C for 7 min on the pilot scale, was provided by the Department of Biotechnology of the Lorena Engineering College (University of Sao Paulo). A portion of the pretreated material was delignified at 100°C for 30 min and with 1% w/v NaOH. The delignification reaction took place in a 20 L rotary reactor fitted with mixing and heating systems (Regmed AUE/20, Regmed Indústria Técnica Ltda., Brazil), using a solid : liquid ratio of 1:10 w/v. The pre-treated and delignified bagasse was filtered through a cloth, and washed seven times to remove the remaining lignin and to reduce the pH. The pulp was dried at 50°C and stored for subsequent chemical analyses and simultaneous saccharification and fermentation.

Simultaneous saccharification and fermentation

Simultaneous saccharification and fermentation took place in 250 mL Erlenmeyer flasks, contained 90 mL of the fermentation medium (nutrients dissolved in a sodium citrate buffer at 50 mM and pH 4.8), 8 g of solids (pre-treated delignified bagasse) and enzyme loads of 10 FPU/g cellulose (Celluclast 1.5 L; 69.50 FPU and 13.70 CBU) and 5% v/v (of the volumetric Celluclast 1.5 L addition) β-glucosidase (Novozym 188; 1340 CBU) preparation, both from Novozymes A/S (Bagsværd, Denmark). The Erlenmeyer flasks were incubated in a rotary shaker at 50°C and 150 rpm. After a 6 h pre-hydrolysis, each Erlenmeyer flask was inoculated with yeast cells and incubated at 37°C and 80 rpm. The enzymatic convertibility of cellulose (ECC) was calculated based in ethanol concentration (Martín et al., 2008):

$$ECC = \frac{E_f - E_i}{C_i \text{ x } 0.57} \text{ x } 100\% \quad (1)$$

Where, E_f is the final ethanol concentration (g/L); E_i is the initial ethanol concentration (g/L); C_i, initial cellulose concentration (g/L). The factor 0.57 is the stoichiometric yield of ethanol from cellulose.

Analytical methods

Samples (10 mL) were filtered in a membrane (0.45 µm) for quantification of the microbial biomass by dry weight. The membrane was heated at 80°C for 24 h until constant weight. The content of the polysaccharides and lignin in the raw material was determined by two-step analytical acid hydrolysis, according to the analytical procedure validated for sugarcane bagasse by Gouveia et al. (2009).

Sugars, carboxylic acids, ethanol and furan aldehydes were quantified by HPLC (Agilent HP 1100, Germany) in an Aminex HPX-87H+ (Bio-Rad, Hercules, CA, USA) column at 60°C, using 5 mM H_2SO_4 at a flow rate of 0.6 mL/min as the mobile phase, and detected using an RI-detector (Agilent). All the experiments were conducted in duplicate. Statistical analysis was performed by analysis of variance (ANOVA) using the software Origin 6.0.

RESULTS AND DISCUSSION

Table 1 shows cell growth (∆X) obtained in fermentation medium by *S. cerevisiae* UFPEDA 1238 and UFPEDA 1324, using the three concentrations of the inoculum (0.4, 4 and 8 g/L). Higher growth was achieved in fermentations with inoculum A (0.4 g/L), independent of strain. Growth with inoculum B (4.0 g/L) and C (8.0 g/L) were much less. This was similar to when *S. cerevisiae* UFPEDA 1324 was used. On the other hand, growth with these inoculum and *S. cerevisiae* UFPEDA 1238 was less than that found for inoculum A, but higher than that found for *S. cerevisiae* UFPEDA 1334.

Glucose consumption (∆S) obtained using two strains and three concentrations of inoculum are shown in Table 1. Higher consumption coincided with lower growth. Glucose can be utilized in different ways by *S. cerevisiae*, depending on the presence of oxygen and carbon sources. In the absence of oxygen, alcoholic fermentation of sugars occurs.

Ethanol production was significantly enhanced as the amount of the inoculum was raised from 0.4 to 4 g/L for the industrial strains *S. cerevisiae* (Table 1): UFPEDA 1238 (95% increase) and UFPEDA 1324 (76% increase). On the other hand, when the amount of inoculum was raised from 4 to 8 g/L ethanol production using the UFPEDA 1324 strain did not increase (Table 1). When the amount of inoculum was raised from 4 to 8 g/L for *S.*

Table 1. Cell growth (ΔX), substrate consumption (ΔS), ethanol production (ΔP), yields ($Y_{X/S}$ and $Y_{P/S}$) and productivities (Q_P) in the fermentations with three inoculum and two industrial strains of *Saccharomyces cerevisiae.*

S. cerevisiae UFPEDA	Inoculum (g/L)	ΔX (g/L)	ΔS (g/L)	ΔP (g/L)	$Y_{X/S}$ (g/g)	$Y_{P/S}$ (g/g)	Q_P (g/L.h)
	0.4	2.31	41.52	11.33	0.06	0.28	1.04
1238	4.0	0.92	64.26	22.08	0.01	0.35	1.84
	8.0	0.57	86.24	37.16	0.00	0.43	3.10
	0.4	1.80	45.71	13.06	0.04	0.29	1.22
1324	4.0	0.12	49.86	22.07	0.00	0.46	1.91
	8.0	0.10	58.07	24.79	0.00	0.43	2.07

cerevisiae UFPEDA 1238, however, ethanol production increased by 64%. Maximum ethanol production (37.16 g/L) and productivity (3.10 g/L.h) were achieved with an initial biomass concentration of 8 g/L in the fermentation of glucose by *S. cerevisiae* UFPEDA 1238. This higher concentration of ethanol was obtained using a high concentration of glucose in the culture medium (100 g/L).

Growth (ΔX) and glucose consumption (ΔS) were used to calculate the fermentation yields ($Y_{X/S}$ and $Y_{P/S}$). Even in fermentations in which there was growth (inoculum A), yields in biomass ($Y_{X/S}$) were lower than 0.06 g/g. The higher yields in ethanol ($Y_{P/S}$) were observed for *S. cerevisiae* UFPEDA 1238 using inoculum C (0.43 g/g) and for *S. cerevisiae* UFPEDA 1324 using inoculum B (0.46 g/g). Productivity (Q_P) varied from 0.85 to 3.18 g/L.h for *S. cerevisiae* UFPEDA 1238 and 0.96 to 2.06 g/L.h for UFPEDA 1324.

Analysis of variance was performed on the productivity obtained from both strains and the three concentrations of inoculum (0.4, 4.0 and 8.0 g/L). These results were significantly different ($F = 36.30$; $\alpha = 0.05$). The analysis of variance between the 0.4 g/L inoculum for both strains, however, showed that the productivity of each was not significantly different ($F = 0.83$; $\alpha = 0.05$).

Likewise, the analysis of variance between the concentration 4.0 g/L inoculum for both strains showed that the productivity did not differ significantly ($F = 0.18$; $\alpha = 0.05$). On the other hand, the analysis of variance between 8.0 g/L inoculum for both strains showed that the productivity of each was significantly different ($F = 24.35$; $\alpha = 0.05$).

Simultaneous saccharification and fermentation of pretreated sugar cane bagasse by *S. cerevisiae* UFPEDA 1238 were carried out with 4 g/L inoculum. This concentration is equivalent to 8 g/L of glucose fermentation, since in the SSF the glucose concentration was about 50 g/L. Figure 1 shows ethanol production, glucose consumption and ECC. There was an accumulation of glucose during the first hours of the process, probably because the cells could not consume glucose at the rate that was released by the enzymes during the early phase of the SSF (Philippidis and Smith, 1995).

Maximum ethanol concentrations coincided with the disappearance of glucose. One reason for the lack of increase in ethanol, after 18 h, can be related to the residual cellulose which seems to be inaccessible to the enzyme (Philippidis and Smith, 1995). Maximum ethanol concentration, enzymatic convertibility of cellulose (ECC) and volumetric productivity were 28 g/L, 76% and 0.93 g/L.h, respectively. Initial cellulose concentration in the pretreated and delignified bagasse was 65.3 g/L. This value was used to calculate ECC according to Equation 1.

Wanderley et al. (2013) in separate hydrolysis and fermentation (SHF) of sugar cane bagasse using 4 g/L inoculum (for 50 g/L initial glucose concentration) reported about 0.9 g/L.h productivity and 24 g/L.h maximum ethanol concentration. However, total time taken was 120 h of hydrolysis and 24 h of fermentation. On other hand, Santos et al. (2012) in an SSF process of pretreated sugar cane bagasse using 1 g/L (18 g/L initial glucose concentration) inoculum found about 25 g/L ethanol, 0.7 g/L.h productivity and 72% of ECC, respectively. Increase of 12, 29 and 5% in ethanol production, productivity and ECC, respectively, was obtained in this work, as compared to that of Santos et al. (2012).

Conclusions

In this work, the concentration of 8.0 g/L of inoculum was selected when the culture medium containing 100 g/L glucose, using *S. cerevisiae* UFPEDA 1238 was used for ethanol production. These conditions favored the process, since it was faster and significantly increased productivity. This work showed that ethanol production from sugar cane bagasse can be increased by use of a high initial biomass concentration.

Conflict of Interests

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

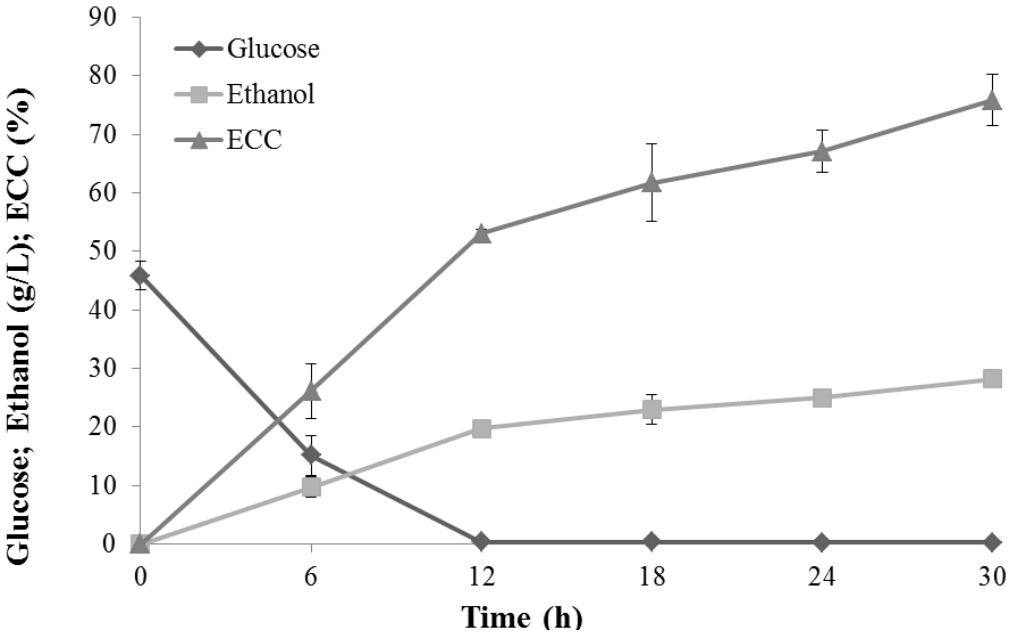

Figure 1. Time course of simultaneous saccharification and fermentation of pretreated sugar cane bagasse using 4 g/L of initial biomass concentration.

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Full Length Research Paper

Development of a novel real-time polymerase chain reaction (PCR) assay by amplification of double target genes for quantitative detection of *Mycoplasma gallisepticum*

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Mycoplasma gallisepticum **(MG) is the most pathogenic and economically significant pathogen in poultry worldwide. Detection of MG is the first and important step for controlling its transmission. A novel real-time polymerase chain reaction (PCR) assay targeting two conservative genes was developed and evaluated carefully in the study. The 16S rRNA and fMG-2 gene fragments were cloned into PCR 2.1 vectors, and recombinant plasmids (r16S21 and rFmg21) were evaluated by PCR, double digestion and nucleotide sequencing. SYBR green real-time PCR was developed using two purified plasmids as templates, and the amplification conditions and reaction systems of real-time PCR were optimized based on specificity, sensitivity and repeatability. A pair of standard curves were assembled for the real-time PCR by detecting two target genes 16S rRNA and fMG-2. The real-time PCR is highly specific to the target genes, with a detection limit of 9 copies/µl. The result of reproducibility shows that the real-time PCR remained consistent. The result of clinical samples demonstrated that the detection rate of the assay was significantly higher than that of the conventional PCR. The double target genes real-time PCR was highly specific, sensitive, and reproducible and could be used on clinical samples from commercial chickens.**

Key words: *Mycoplasma gallisepticum*, real-time PCR, double target genes, chronic respiratory disease (CRD).

INTRODUCTION

Mycoplasma gallisepticum (MG) is an important infectious respiratory pathogen within chicken farming plant (Leigh et al., 2010). It can trigger chronic respiratory disease (CRD) in chickens and cause serious economic losses for poultry farming (Osman et al., 2009). CRD shows a variety of symptoms including rales of respiratory tract, cough, tracheitis, air sacculitis and maudlin, which often reduce the feed and egg production efficiency (Raviv et al., 2008). MG can be transmitted horizontally by direct or indirect contact manner through the respiratory tract. However, this pathogen does not survive outside of the host for extended periods

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Table 1. Primers used for real-time PCR analysis and gene cloning.

Primer	Gene	Real-time RT-PCR Sequence(5′-3′)	Cloning Sequence(5′-3′)
16sRNA(F)	*16sRNA*	TGGGGGTGGATTACCTCCTTTCT	GCTAACCGCAAGGAAG
16sRNA–(R)		TTTTATGGGTTTAACTTTTCTTGCT	GAATAGAATCCGACCAAC
fMG-2(F)	*fMG-2*	AATCTTTTGTGAGTAGGTGTTGGC	CATTCAAAGATTTCGTG
fMG-2(R)		AATAGTTTTGACGTATCATTTAGTGC	CCAGCGATACTCCTAA

(Sprygin et al., 2011). Mortality due to CRD is lower than other avian diseases, but the infection rate is very high as compared with other diseases. Within intensive livestock farming, infection by CRD often triggers Newcastle disease, avian influenza, infectious bronchitis and colibacillosis, these diseases may lead to more severe problems (Stipkovits et al., 2012).

To date, the accurate and rapid diagnosis for MG is essential to prevent and control the infection by this pathogen. Many detection methods were developed and researched recently, including serology, culture, and molecular identification (Garcia et al., 2005; Ghorashi et al., 2010; Kahya et al. 2010; Zhang et al. 2011). Although isolation of mycoplasmas is the gold standard way for determining MG, time-consuming for this method limits its wide application within poultry production (Levisohn and Kleven, 2000). Serological assays for detecting MG show higher efficient and rapid, such as plate agglutination test, haemagglutination inhibition and enzyme-linked immune-sorbent assay (ELISA) (Kempf and Gesbert, 1998; Kempf et al., 1994), but these methods have other limitation for detecting MG infection, including hysteresis of antibody generation and cross-reactions with other pathogens (Kempf et al., 1997). DNA detection is an alternative to conventional culture and serology, of these methods real-time PCR is a more sensitive and rapid assay than conventional PCR detection (Mekkes and Feberwee, 2005). In this study we report the development of a highly specific and sensitive SYBR green real-time PCR assay for detecting 16S rRNA and fMG-2 genes of MG. The novel method was embodied for detecting double conservative genes.

MATERIALS AND METHODS

MG strains and DNA preparation

Six mycoplasma strains were used in this study: *M. gallisepticum* S6 strain was used as template for development of real-time PCR, *Mycoplasma synoviae* WVu 1853 strain, *Mycoplasma iowae* 695 strain, *Mycoplasma hyorhinis* BTS-7 strain, *Acholeplasma oculi* PG 8 strain, *Mycoplasma arginini* G230 strain (China Institute of Veterinary Drug Control), *Escherichia coli* O157:H7 strain, *Salmonella enteritidis* C50041 strain, and *Vibrio parahaemolyticus* VP12 strain (our lab) were used to validate the specificity of real-time PCR. The genomic DNA was extracted (DNeasy Blood and Tissue Kit, Qiagen®, of Hilden, Germany) following the manufacturer's instructions from the culture these bacteria, and aliquots of the DNA were stored at -80°C for real-time PCR assay.

Primers for real-time PCR

The real-time PCR assay was designed based on the sequences of 16S rRNA (FJ468422) and fMG-2 (AF075588) genes of MG S6 strain. The primers were designed with primer 3.0 online (http://www.simgene.com/Primer3), and the specificity of primers was assessed using BLAST online (http://www.ncbi.nlm.nih.gov/BLAST/). The cloning primers were designed using primer 5.0 software (Premier Inc., Canada). The primers used are given in Table 1.

Preparation of standard DNA controls

16S rRNA and fMG-2 genes were cloned using specific primers from MG S6 strain. The reaction was carried out in a 25 µl reaction mixture containing 2.5 µl of 10xPCR buffer, 1.0 µl of dNTPs (2.5 pmol·µL^{-1}), 0.5 µl of Taq DNA polymerase enzyme (5 U·µL^{-1}, TaKaRa, Dalian, China) and 3 µl of template, primers to a final concentration of 0.2 µM and nuclease free water was added to make 25 µl. The reaction conditions used are 95°C for 15 min; and 30 cycles of 95°C for 30 s followed by the reaction specific annealing 46°C for 1 min and extension 72°C for 50 s, and finally extension at 72°C for 5 min. The fragments identified as of 16S rRNA and fMG-2 genes were purified using MiniBEST Agarose Gel DNA Extraction Kit Ver.3.0 ® (TaKaRa, Dalian, China). Fragments cloned were ligated into vector pCR 2.1 vector (named r16S21 and rFmg21) and transformed into competent *E. coli* DH5α cells. Finally, *E. coli* DH5α cells were cultured on Luria-Bertani plates containing X-gal and IPTG; the white colonies were selected and analyzed using PCR and digested using *Bam*H and *Xho* for r16S21 plasmid, *Xho* and *Hind* β for rFmg21 plasmid. The plasmids of r16S21 and rFmg21 were subjected to DNA sequencing in Genscript® (Genscript Inc. Nanjing, China).

Standard curves for Real time PCR

The standard curves were drawn by real-time PCR, which was performed using templates of 16S rRNA and fMG-2 recombinant plasmids. The reaction system was a 20 µl mix containing 10 µl of SYBR Premix Ex Taq Ⅱ 2xMix (TaKaRa, Dalian, China), 0.4 µl of Rox Reference Dye Ⅱ (TaKaRa, Dalian, China), primers to a final concentration of 0.4 µM, 6 µl of water, and 2 µl of template (40 ng/µl). Each real-time RT-PCR reaction was performed using a Gene Amp 7500 thermocycler (Applied Biosystems, Carlsbad, California, USA) with the following PCR conditions: 50°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 s and annealing at 60°C for 34 s. The quantitation and detection limit of real-time PCRs were determined by 3 independent runs of each reaction, using 10-fold serial dilutions (10^9 to 10^5 copies per reaction) of r16S21 and rFmg21 plasmid as templates. The standard curves were generated by plotting the mean C_T values vs. $\log_{10}$ of the plasmid copy numbers of the three independent runs.

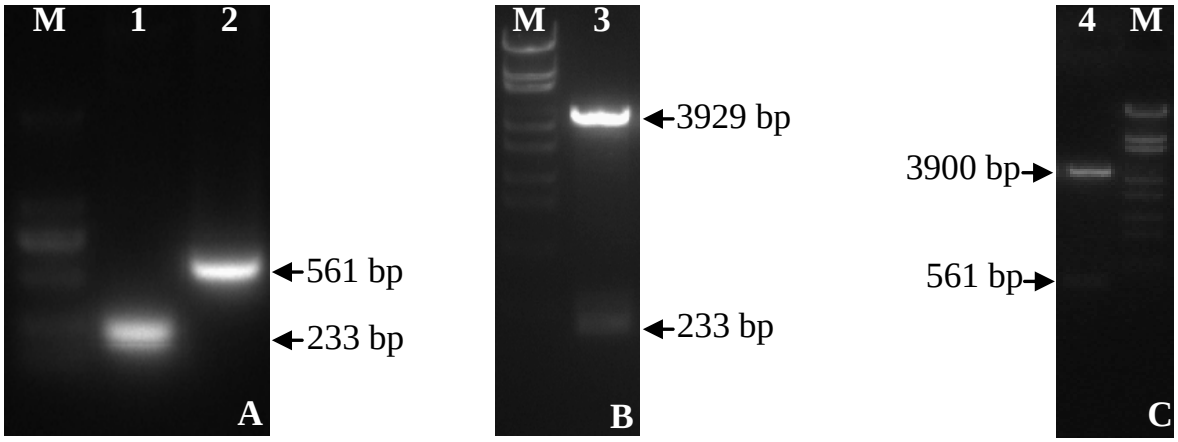

Figure 1. Results of PCR evaluation and restriction enzyme digestion for recombinant 16S rRNA and fMG-2 plasmids. Fragments of 16S rRNA and fMG-2 were ligated into vector pCR 2.1 vector, and PCR were performed to evaluate the recombinant plasmids. The result of Figure 1A shows that fragments of 233 and 561 bp were amplified for 16S rRNA and fMG-2, respectively. The Figure 1B and 1C shows the results of restriction enzyme digestion, 16S rRNA by using BamH Ⅰ and Xho Ⅰ, fMG-2 by HindⅢ and Xho Ⅰ.

Specificity test

To verify the specificity of the developed real-time PCR assay, each reaction was performed using genomic DNA extracts from microorganisms including *M. gallisepticum* S6 strain*, M. synoviae* WVu 1853 strain, *M. iowae* 695 strain, *M. hyorhinis* BTS-7 strain, *Acholeplasma oculi* PG 8 strain and *M. arginini* G230 strain, *E. coli* O157 strain, *Salmonella pullorum* S06004 strain, *Campylobacter jejuni* NCTC 11168 strain, *V. parahaemolyticus* isolate VP12 and mixtures of all these organisms. The real-time PCR were performed according to the procedure and setup as mentioned above.

Sensivity test

The genomic DNA was extracted (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany) from *M. gallisepticum* S6 strain, and its concentration was determined using BioPhotometer plus (Eppendorf Inc., Germany). Serial ten-fold dilutions of template were made for confirming the minimum detection limit of the real-time PCR assay developed. Any reaction whose C_T value was under 35 was considered positive and higher C_T values were considered negative.

Reproducibility test

For the reproducibility of the real-time PCR assay, the test of variation within-run and between-run were performed for 3 reactions using recombinant plasmids (r16S21 and rFmg21) as templates.

Application of the real-time PCR for clinical samples

In this study, 30 samples of chicken swabs were collected from five farms (six swabs per farm) in Anhui province, from birds showing clinical symptoms of chronic respiratory disease (CRD). Real-time PCR were used to detect MG in these samples and normal PCR assay as control. 10 samples from apparently healthy birds were used as negative controls.

RESULTS

Standard DNA controls

The 16S rRNA and fMG-2 genes were cloned from *M. gallisepticum* S6 strain, and these 2 recombinant plasmids were determined by using PCR, double digestion (Figure 1) and DNA sequencing array (data not showed).

Standard curves

Standard curves were made for the real-time PCR by detecting 2 target genes (16S rRNA and fMG-2). Each reaction's standard curve was determined by 3 independent runs of each reaction using 10-fold serial dilutions (10^9 to 10^5 copies per reaction) of the standard DNA control for 2 target genes. The mean C_T values, the linear equations and the R-squared values of the real-time PCRs standard curves are shownin Table 2. The curves were depicted as Figure 2, and constructed by 7500 software, version 2.0.1 (Applied Biosystems, Carlsbad, California, USA).

Specificity of the real-time PCR

The results of real-time PCR specificity are given in Figure 3A and 3B. The reactions were positive by detecting genomic DNA of *M. gallisepticum* S6 strain and mixtures of these 10 organisms; other reactions were negative including those of *M. synoviae* WVu 1853 strain, *M. iowae* 695 strain, *M. hyorhinis* BTS-7 strain, *Acholeplasma oculi* PG 8 strain, *E. coli* O157 strain, *S.*

Table 2. Summary of the mean C_T values, the linear equations and the R-squared values of the real-time PCRs standard curves.

MG strain real-time PCR (target gene)	Mean C_T values of template $\log_{10}$ copy number					Linear equation	R-squared
	5	6	7	8	9		
16sRNA	14.714	11.337	7.864	4.873	4.391	Y=-2.858X+29.316	R^2=0.988
fMG-2	15.672	12.306	8.474	5.143	4.964	Y=-2.663X+27.296	R^2=0.990

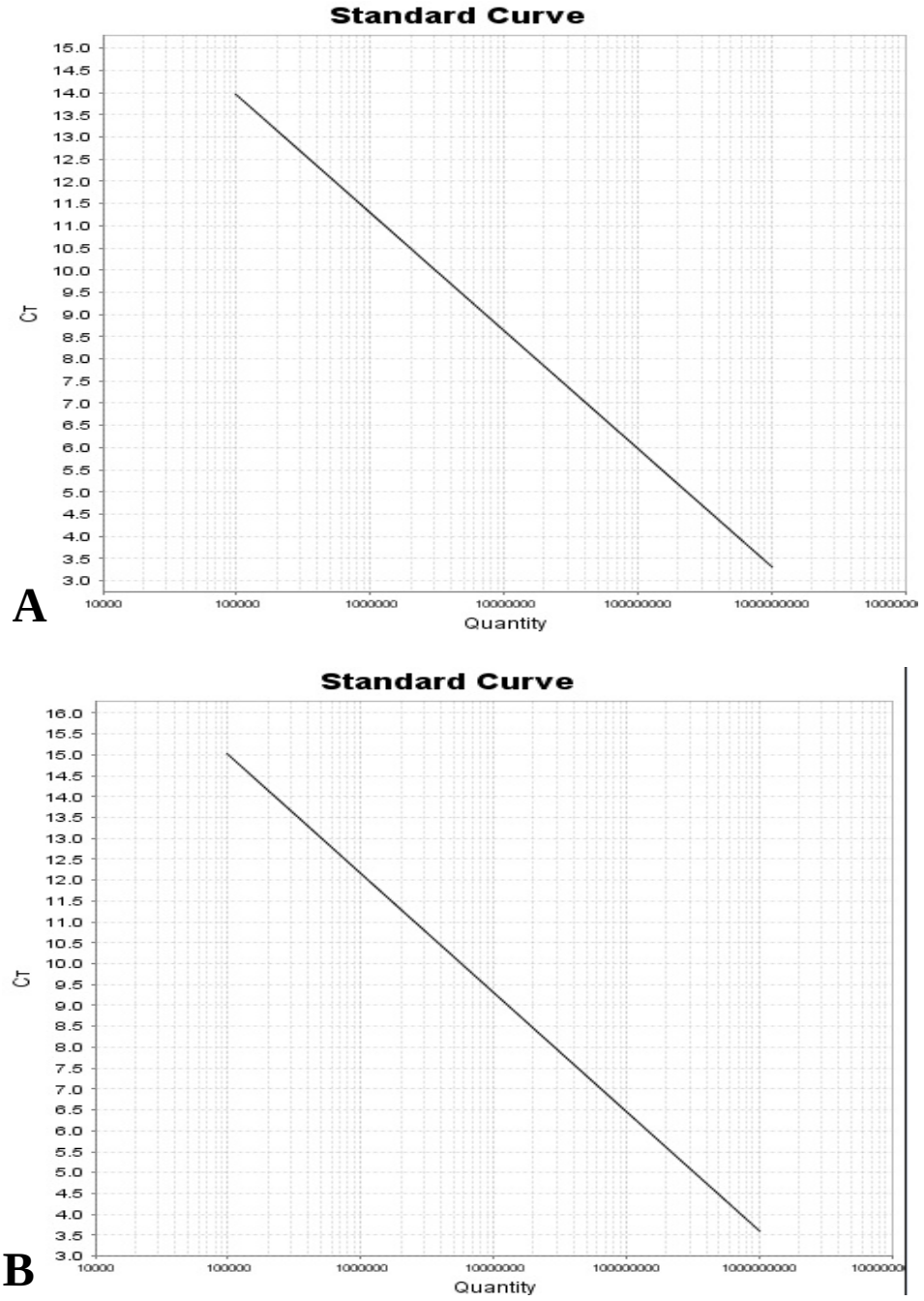

Figure 2. Standard curve of real-time PCR for recombinant plasmids for 16S rRNA and fMG-2. The standard curves were determined using 10-fold serial dilutions (10^9～10^5 copies per reaction) of the standard DNA control for 2 16S rRNA (Figure 2A) and fMG-2 (Figure 2B) genes by ABI 7500 software Version 2.0.1.

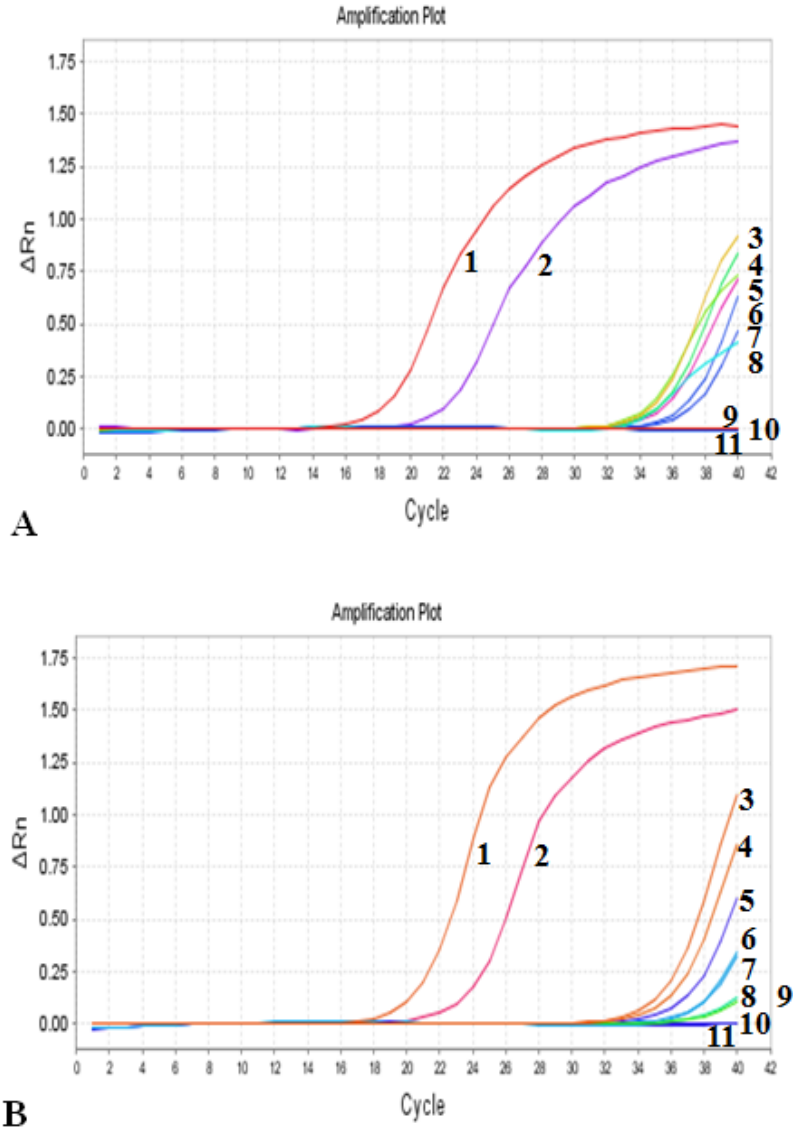

Figure 3. Dynamic curves of real-time PCR for determining *M. gallisepticum* specificity. The real-time PCR were performed to detect 16S rRNA (Figure 3A) and fMG-2 (Figure 3B) genes by using these genomic DNA as templates, including *M. gallisepticum* S6 strain (Curve 1), *M. synoviae* WVu 1853 strain (Curve 3), *M. iowae* 695 strain (Curve 4), *M. hyorhinis* BTS-7 strain (Curve 5), *Acholeplasma oculi* PG 8 strain (Curve 6), *E. coli* O157 strain (Curve 7), *S. pullorum* S06004 strain (Curve 8), *C. jejuni* NCTC 11168 strain (Curve 9), *V. parahaemolyticus* isolate VP12 (Curve 10), *M. arginini* G230 strain (Curve 11) and mixtures of these 10 organisms (Curve 2).

pullorum S06004 strain, *C. jejuni* NCTC 11168 strain, *V. parahaemolyticus* isolate VP12 and *M. arginini* G230 strain. These results show that the real-time PCR assay is highly specific to the target genes and no cross-reactivity was observed for other control microorganisms.

Sensitivity of the real-time PCR

The concentration of genomic DNA of *M. gallisepticum* S6 strain was 40 µg/µl; amount to 9.25×10^{7} copies/µl. The sensitivity for *M. gallisepticum* was determined using genomic template DNA extracted from nine 10-fold dilutions of *M. gallisepticum* S6 strain. The detection limit of the developed real-time PCR was 9 copies/µl (Figure 4A and B).

Reproducibility of the real-time PCR

The reproducibility of the real-time PCR assay was determined through the test of variation within-run and

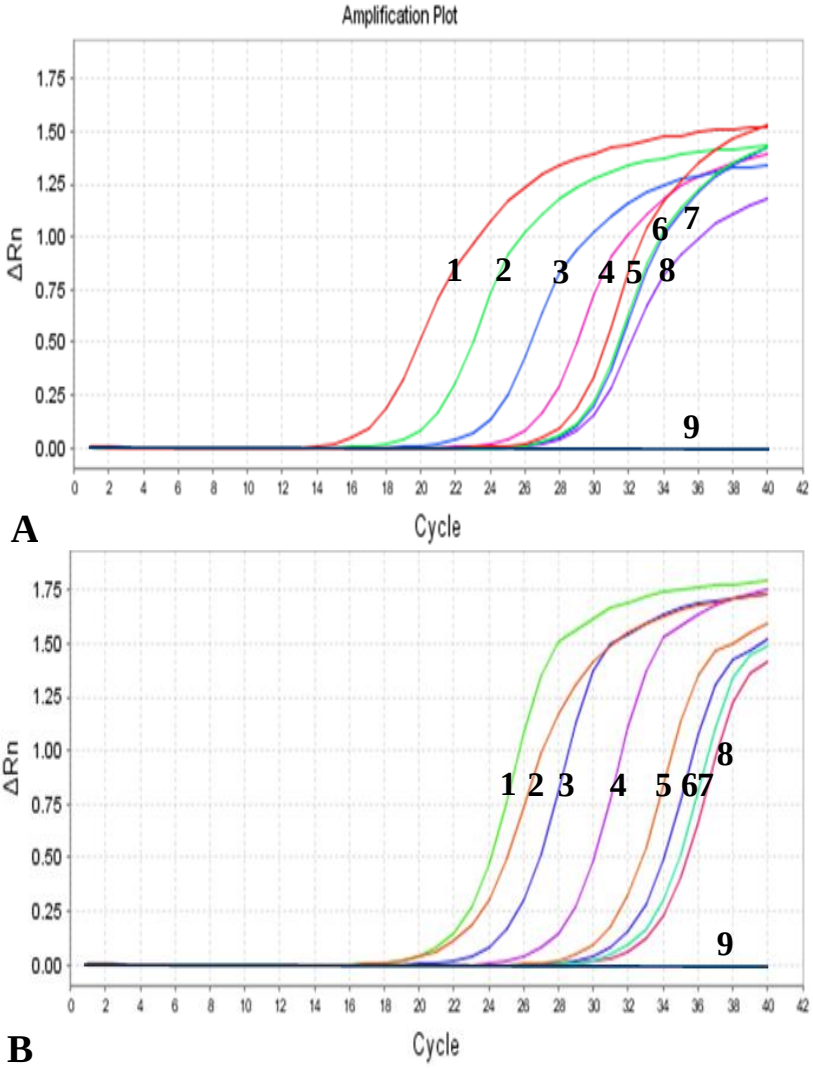

Figure 4. Dynamic curves of real-time PCR for determining *M. gallisepticum* sensitivity. The sensitivity of real-time PCR were determined for 2 target genes, 16S rRNA (Figure 4A) and fMG-2 (Figure 4B), and genomic template DNA were extracted from nine 10-fold (Curves 1 to 9) dilutions of M. gallisepticum S6 strain (9.25×10^7 copies/μl).

between-run using recombinant plasmids (r16S21 and rFmg21) as templates. All reactions remained consistent, which shows that the real-time PCR is repeatable (data not shown).

Detection of the real-time PCR for clinical samples

Out of 30 suspected samples, 17 (56.7%) were positive by real-time PCR, and six samples (20%) were positive through conventional PCR assay. There are no positive out of the 10 samples from chickens without symptoms by using both methods. These results show that the developed real-time PCR has higher sensitivity accuracy and have applicability on clinical samples.

DISCUSSION

In China, MG is the most economically significant poultry mycoplasma, and gives rise to respiratory disease in chickens, turkeys and other avian species. Control of CRD has generally been based on the eradication of the pathogen from breeder flocks and the maintenance of mycoplasma-free status in the breeders and their progeny by biosecurity practices. Serology is the primary method for flock supervisory screening. Sera commonly are examined for antibodies using the serum plate agglutination (SPA) test, a hemagglutination-inhibition (HI) test and an enzyme-linked immunosorbent assay (ELISA) test (Ben Abdelmoumen Mardassi et al., 2008; Raviv et al., 2008). The SPA test is rapid, sensitive and

inexpensive but may generate nonspecific results. The HI test is less sensitive but more specific than the SPA test. The ELISA test is more sensitive than the HI test and more specific than the SPA test. However, many studies showed that serological assays have some drawbacks (Sprygin et al., 2010).

The primary goal of the present study was to develop a real-time PCR approach with double target genes to detect *M. gallisepticum* sensitively in a quantitative manner from clinical setting. Genes 16S rRNA and fMG-2 were two important markers for detecting MG. Sequence data of the 16S rRNA genes have proved to be very useful in studying the phylogeny of MG (Olsen and Woese, 1993). The 16S rRNA nucleotides are convenient for rapid sequence analysis and the presence of universal regions enables its amplification by PCR for subsequent analysis. Another important advantage of the 16S rRNA gene is that it is not prone to horizontal gene transfer (Raviv et al., 2007), and that polymorphism between copies of the gene in the chromosome are not common, due to recombination by gene conversion. Gene of fMG-2 was a conservative marker for detecting MG, some developed PCR approach based on this gene have higher specificity. In this study, these 2 genes were determined separately using real-time PCR.

At present, some MG species-specific PCR, MG real-time PCR, PCR-RFLP and oligonucleotide probe techniques are available (Callison et al., 2006; Jarquin et al., 2009; Kahya et al., 2010; Lierz et al., 2008; Mardassi et al., 2005; Raviv et al., 2008; Sprygin et al., 2010). During the acute stage of the infection the number of MG in the upper respiratory tract is high, but in chronic infection the number of organisms is much lower and routine methods may not detect it (Levisohn and Kleven, 2000). In some situations it may be very difficult to isolate pathogenic mycoplasma consistently from infected flocks. These instances include chronic MG and MS cases and infections with strains of low pathogenicity (Lam and Lin, 1984). The present assay can be applied to the detection M. gallisepticum from clinical samples, and its accuracy was higher than other molecular methods for based on 2 target genes. The specificity and sensitivity of the real-time PCR were evaluated, the results show that MG can be differentiated from other micrograms, including of *M. synoviae* WVu 1853 strain, *M. iowae* 695 strain, *M. hyorhinis* BTS-7 strain, *Acholeplasma oculi* PG 8 strain, *E. coli* O157 strain, *S. pullorum* S06004 strain, *C. jejuni* NCTC 11168 strain, *V. parahaemolyticus* isolate VP12 and *M. arginini* G230 strain. The detection limit of the developed real-time PCR was 9 copies/µl. The real-time PCR assay was evaluated for the ability to detect MG in clinical specimens using 30 samples, and the results indicated that the developed real-time PCR has higher sensitivity than conventional PCR assay on clinical samples. In summary, we have developed a sensitive and specific real-time PCR assay for the detection of the *M. gallisepticum* in clinical samples from commercial poultry.

Conflict of Interests

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

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Short Communication

Melanoides tuberculata as intermediate host of *Centrocestus formosanus* (Nishigori, 1924) in Tunisia

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***Melanoides tuberculata* that naturally harbored trematode larvae were collected at the irrigation system in Ain Soltan's oasis (southwest of Tunisia), during malacological surveys conducted from January to December 2013. From 2160 specimens of *M. tuberculata* collected, 31 (1.4%) were infected by the cercariae of *Centrocestus formosanus* (Digenea: Heterophyidae). The parasite was present in snails larger than 19 mm. This is the first report of *C. formosanus* in *M. tuberculata* in Tunisia.**

Key words: *Centrocestus formosanus*, *Melanoides tuberculata*, Tunisia, intermediate host.

INTRODUCTION

Snail species are the first intermediate host for most of parasitic trematodes that provoke diseases such as heterophyidiasis, schistosomiasis, and fascioliasis. *Melanoides tuberculata* (Müller, 1774) is a fresh water snail, which lives in the bottom of water and in abundance in the irrigation system in Ain Soltan's oasis in the south west of Tunisia. It has been recorded as the first intermediate host of the tremadote *Echinochasmus japonicus*, which is a trematode of birds and mammals, and belongs to the Echinostomatidae. (Cheng and Fang, 1989), which occasionally may infect human beings (Sayasone et al., 2009). Furthermore, *M. tuberculata* also harbors the trematode *Clonorchis scinensis*, agent of the clonorchiasis disease (Kino et al., 1998). Xiphidiocercariae, aquatic larval stages of some trematodes are considered a potential instrument for biological control of mosquitoes. This tramatodes have been described from seven Egyptian snails, including *M. tuberculata* collected from Giza and Qualiobyia (Wanas et al., 1993). The aim of this study was to determine the cercarial fauna of *M. tuberculata* snail and their role in transmitting diseases in Ain Soltan's oasis (south-western Tunisia).

MATERIALS AND METHODS

M. tuberculata snails were collected manually from irrigation system in the oasis of Ain Soltan in the governorate of Gafsa, Tunisia (34°27'13"N 8°47'44"E) from January to December 2013 to find the cercariae,.

Collected snails were transferred in plastic bags to the laboratory, where they were thoroughly cleaned and maintained for several

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Figure 1. *Melanoides tuberculata* snail from Ain Soltan's oasis of Southwestern Tunisia.

Figure 2. The cercariae of *Centrocestus formosanus.*

weeks in aquaria with a thin substrate of sand and dechlorinated water and provided with green algae for feeding.

They were counted, measured using caliper and examined for cercariae by crushing method. In this method, shell of snails was broken and soft tissues were placed between two slides then squashed. Collected cercariae were studied live, unstained and stained with vital stains, and fixed in 5% formalin, preserved in 70% ethanol, cleared in lactophenol and stained with a 10% Rose Bengal solution. Body proportions were measured by ocular micrometer.

RESULTS

In all, 2160 specimens of *M. tuberculata* (Figure 1) were collected and examined. Cercaria emerged from 31 *M. tuberculata* specimens (1.4%). Cercaria was preliminarily characterized as belonging to the family Heterophyidae, genus *Centrocestus* (Figure 2). Cercariae tended to emerge during the morning, in small numbers, and were very active.

Cercariae presented Piriform body, morphologically agreeing with the descriptions and drawings of *C. formosanus* from *M. tuberculata* of China, Hong Kong, Taiwan, and Mexico (Chen, 1948; Arizmedi, 1992), and *S. newcombi* of Hawaii (Martin, 1958).

Measurements (given in µm, with average in parentheses) of 20 fixed and stained specimens, without cover glass pressure, were: body length of 114-156 (141); body width of 77-104 (84); oral sucker length of 29-38 (35); oral sucker width of 26-34 (39); pharynx diameter of 13-16 (14); ventral sucker diameter 20-25 (23); tail length of 115-156 (132); tail width pf 16-22 (18).

Under test conditions, the cercariae of *C. formosanus* swam in more or less straight lines. *M. tuberculata* whose size shell length is less than 19 mm showed no larval forms of *C. formosanus*. The parasite is only present in snails larger than 19 mm. Infection rate increases exponentially with snail body size (Table 1 and Figure 3). The regression fit is:

Infection rate = 0.107 - 7.523*Shell size.

The correlation coefficient was R = 0.999 and this function explains 99.984% of variance in infection rate.

DISCUSSION

The life cycle of *C. formosanus* has primarily been elucidated in Taiwan, where the developmental stages and the participation of thiarids mollusks in its transmission have been described (Nishigori, 1924). Since then, several studies have confirmed the participation of *M. tuberculata* as an intermediate host of *C. formosanus* in different countries, such as China (Chen, 1948; Chao et al., 1993), Japan (Yanohara, 1985; Yanohara, et al., 1987), Mexico (Salgado-Maldonado et al., 1995; Scholz et al., 2000; Scholz and Salgado-Maldonado, 2000), Malaysia (Bayssade-Dufour et al., 1982), Taiwan (Lo and Lee, 1996a, b), India (Madhavi et al., 1997), United States (Tolley-Jordan and Owen, 2008), Venezuela (Hernandez et al., 2003), Iran

Table 1. Variation of infection rates according the size of *Melanoides tuberculata.*

Size of snail (mm)	<3	<10	<15	<20	<25	<30	<35	<40	Total
Snail samples	248	415	353	304	629	169	37	5	2160
Snail samples infected	0	0	0	0	8	10	8	5	31
Infection rate (%)	0	0	0	0	1.3	5.9	21.6	100	1.4

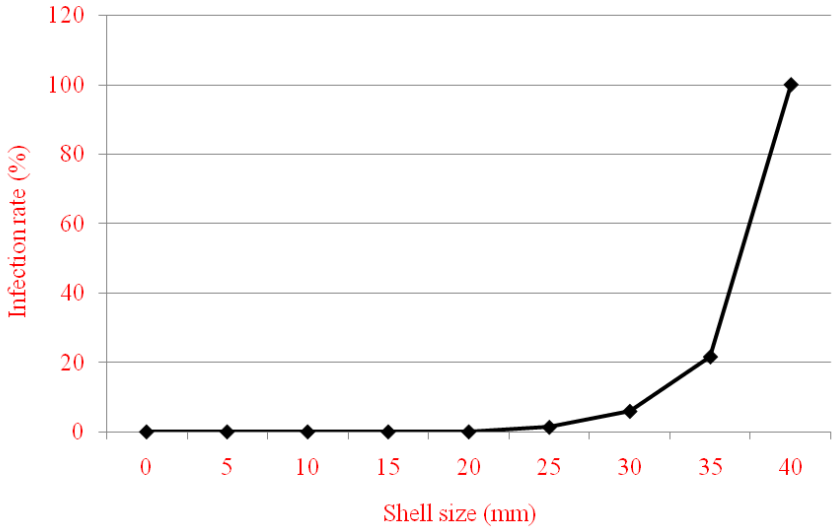

Figure 3. Infection rate = f (Shell size).

(Farahnak et al., 2005), Colombia (Velásquez et al., 2006; Vergara and Velásquez, 2009) and Brazil (Pinto and Melo, 2010). Despite these reports, the possible involvement of *M. tuberculata* in the transmission of *C. formosanus* has still not been reported in Tunisia.

In the present study, the participation of *M. tuberculata* in the biological cycle of C*. formosanus* in Tunisia is confirmed, but the natural definitive host of *C. formosanus* in Ain Soltan's oasis remains unknown. Knowing that there are freshwater fish and birds associated to *M. tuberculata*. Given that this snail is widespread in Tunisia and participates in the life cycle of *Centrocestus* in the country, the impact of introducing and spreading these thiarids around the country must be better evaluated, with an aim toward preventing future cases of Centrocestiasis. Centrocestiasis is a gill trematode disease of numerous cultured and wild fish species that is caused by a digenetic trematode *Centrocestus formosanus*.

In this study, only snails larger than 19 mm are infested, implying that the infestation rate increases with increase in the size of *M. tuberculata*. Also, in Texas the parasite is usually only present in snails larger than 17 mm (Mitchell et al., 2000).

Conflict of Interests

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

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