African Journal of Microbiology Research

Volume 8 Number 47, 19 November, 2014
ISSN 1996-0808

Academic Journals

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African Journal of Microbiology Research

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academicJournals

Vol. 8(47), pp. 3811-3814, 19 November, 2014
DOI: 10.5897/AJMR2014.6785
Article Number: 5178F4548990
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

The use of Artemisia tea and occurrence of single nucleotide polymorphisms in the *PfATPase6* gene

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Received 18 March, 2014; Accepted 18 November, 2014

The herb *Artemisia annua* L. was used in ancient Chinese medicine to treat malaria and fevers. In a Ugandan community (Wagagai flower farm) located at the shores of Lake Victoria in Entebbe Municipality, tea infused with dried leaves of this herb (Artemisia tea) is drank by employees. The effect of drinking Artemisia on changes (single nucleotide polymorphisms, SNPs) in *Plasmodium falciparum* candidate resistance genes is not known. This study therefore investigated the use of Artemisia tea and the occurrence of SNPs in the PfATPase6 gene. A section of the *PfATPase6* gene of 1940 bps was amplified by polymerase chain reaction (PCR) and the amplicons purified, followed by sequencing. Using CLC MainWorkBench software, the sequences were aligned and with *P. falciparum* 3D7 as reference sequence, SNPs were manually inspected in the sequences (n=17). Single nucleotide polymorphisms were observed at two positions from the 17 sequences: SNP T1707A occurring in 2/17 (11.8%) of the sequences and SNP T2694A in (10/17) 58.8% of them. This study identified two SNPs in the PfATPase6 gene of *P. falciparum* isolates obtained from employees of Wagagai flower farm who were using Artemisia tea to protect themselves against malaria. However, based on studies done elsewhere, these SNPs where not associated with artemisinin resistance.

Key words: Artemisia tea, *Pf*ATPase6 gene, single nucleotide polymorphisms.

INTRODUCTION

Artemisia annua L. has been used in herbal treatment of intermittent fevers and malaria by the Chinese for centuries; the active ingredient, artemisinin was isolated from this herb in 1971 (Hsu, 2006). Artemisinin based combination therapies (ACTs) was recommended and is currently used in the treatment of uncomplicated malaria (WHO: Guidelines for treatment of malaria, 2010). However, because ACTs are relatively expensive and may not be readily available in many areas with malaria in Africa, herbal preparations of *A. annua* L. is currently being promoted to treat malaria (Willcox et al., 2011). In a Ugandan community, tea infused with dried leaves of *A.*

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annua L. (Artemisia tea) is drank to cure or protect against malaria and it has been shown to be efficacious (Ogwang et al., 2011). However, the quantity of artemisinin in the tea is low; plants grown in East Africa yield low quantities of artemisinin ranging from 0.2 to 0.35% (Wilcox et al., 20l1), as compared to those grown professionally in Europe with a quantity of between 0.5 and 0.70% of artemisinin (Mueller et al., 2000). Artemisia tea prepared with 5 g of dried leaves contains about 60-70 mg of artemisinin and if administered for 5 days, reaches 320 mg, this is in contrast to the usual clinical dose of the artemisinin derivatives (artesunate or artemether) which is between 500 and 1000 mg total within 2 - 6 days of treatment (de Vries and Dien, 1996).

There is therefore concern that the low quantity of artemisinin in the preparation may not be sufficient to clear all *P. falciparum*, leaving behind parasites which may develop resistance to artemisinin (WHO: Position statement on the effectiveness of Non-Pharmaceutical Forms of *A. annua* L. against malaria, 2012). Resistance to antimalarials is associated with SNPs in *P. falciparum* genes; *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) to Chloroquine resistance (David et al., 2000; Wallis et al., 2004), Dihydropteroate synthetase (dhps) and dihydrofolate reductase (dhfr) genes to sulphadoxine-pyrimethamine (Pedro et al., 2005). Earlier studies suggested PfAPase6 gene to modulate artemisinin resistance (Uhlemann et al., 2005; Jambou et al., 2005), however currently polymorphisms in the Kelch and cysteine protease falcipain-2 genes have also been implied (Ariey et al., 2014). This study being reported was done in 2010, at that time the roles of Kelch and cysteine protease falcipain-2 genes to artemisinin resistance had not yet been identified. The main objective of this study was to determine whether the use of Artemisia tea is associated with Single nucleotide polymorphisms which may occur in the PfATPase6 gene in *P. falciparum.*

MATERIALS AND METHODS

Sample collection

The study was done in Wagagai flower farm, located at the shores of Lake Victoria in Entebbe. *P. falciparum* isolates were from adults, who participated in Artemisia tea study performed in 2010; samples were collected with consent from farm management and workers and also the study received ethical clearance from the Higher Degree Committee of Makerere University (Ogwang et al., 2011). Participants were all employees of the farm; here Artemisia tea was administered at least once a week by farm management, as a preventive measure against malaria. This had been going on for a period of about four years. Samples were collected from a health centre which belongs to the farm and is located within its premises. Thick Giemsa-stained blood smears and blood spots on 3 mm Whatman 903® FTA card were obtained from those who showed fever and related symptoms of malaria. All patients positive for malaria were treated with Artemether-lumefantrine. FTA cards (n=46) from malaria positive patients by microscopic examination were randomly selected from those archived for genotyping. Another 12 FTA cards from malaria positive patients by microscopy examination were obtained from a clinic in Entebbe town from patients who were not exposed to Artemisia tea.

Plasmodium falciparum speciation

DNA was extracted from blood spots on 58 FTA cards (n = 46 from Wagagai, n = 12 from the clinic) by chelex (Sigma-Aldrich), in 100 µL of sterile water as described by Dokomajilar et al. (2007). A nested PCR was used to amplify about 200 bp of the 18S small subunit ribosomal DNA. Primers used were rPLUf: 5'- CCT GTT GTT GCC TTA AAC TTC 3'/ rPLUf: 5'- TTA AAA TTG TTG CAG TTA AAA CG 3' for the primary reaction and rFLUf: 5'- TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT 3'/ rFLUr: 5'- ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC 3' (Dokomajilar et al., 2007), for the nested reaction. The amplifications were performed in 20 µl reaction containing, 0.5 µM of forward and reverse primers, 200 µM dNTPS, 1x Phusion HF Buffer containing 1.5 mM $MgCl_2$, 0.02 U of Phusion™ DNA polymerase (FINNZYMES Oy), 3 µl DNA template and 12.2 µl PCR grade sterile water for the primary PCR reaction; 1 µl of amplicons from the primary reaction and 14.2 µl PCR grade sterile water for the nested reaction. Genomic DNA from *P. falciparum* 3D7 was used as a positive control and PCR grade water as negative control. Thermocycler (Techno® TC-412) was used and the conditions were: 98°C for 1 min, 35 cycles of 98°C for 10 s, 64°C for 30 s for primary reaction, for nested reaction 68°C for 30 s annealing, 72°C for 30 s, and 72°C for 5 min for all reactions. The products were electrophoresed on 2% agarose gel stained in ethidium bromide with 1xTBE (Sigma Aldrich) as the running buffer and visualized using uvitec illuminator then documented.

PfATPase6 gene analysis

Amplification of *PfATPase6* gene was performed on only those samples confirmed to be positive for *P. falciparum* by the *P. falciparum* specific PCR (n=35 from Wagagai, n=7 from the clinic). A section of the of *PfATPase6* gene containing bases 1138 to 3078 (1940 bps), where SNPs were reported to occur (Ferreira et al., 2008; Maman et al., 2009) was amplified by PCR. The primers used for this PCR reaction were 5F: 5'- ACCGTGTTTCATTTGTTTAGAG 3'/10R: 5'-TGTGCTGGTAATCCGTCAG 3' (Ferreira et al.2007). The amplification was done in 25 µl PCR reaction mixture containing, 0.1 µM of forward and reverse primers, 200 µM dNTPS, 1xPhusion HF Buffer containing 1.5 mM $MgCl_2$, 0.04 U of Phusion™ DNA polymerase (FINNZYMES Oy) and 3 µl of DNA template. The PCR conditions were:9 8°C for 1 min, 35 cycles of 98°C for 10 s, 62°C for 30 s, 72°C for 90 s and 72°C for 10 min. The amplicons were electrophorised on 1% agarose gel stained with ethidium bromide in 1xTBE running buffer and visualized using uvitec illuminator. Amplicons of the correct size (1940) were cut out using a sterile scalpel and DNA extracted and purified using GENJET™ Gel Extraction Kit (Fermentas). A total of 25 purified amplicons (n=16 from wagagai, n=9 from the clinic) were selected and sent to Macrogen Korea (http://dna.macrogen.com) for sequencing. The sequences were aligned with *P. falciparum* 3D7 sequence obtained from Plasmodium data base with accession number of PFAO310C, using the CLC MainWorkBench software version 6.0.2 (http://www.clcbio.com) and SNPs manually scored.

RESULTS

After editing the sequences for any ambiguity, 17 out of the 25 were consistent, and only these 17 sequences were inspected for SNPs. Two SNPs were observed (Table 1).

Table 1. A PfAPase6 gene substitution from *P. falciparum* isolates from Entebbe.

Nucleotide position	Normal allele	Mutant allele	Mutation type	Normal amino acid	Mutant amino acid	n/N
1707	T	A	Synonymous	N	K	2/17
2694	T	A	Non-Synonymous	I	I	10/17

n = number of samples with mutant allele; N = total number of sampled sequenced and inspected for SNPs.

A synonymous polymorphism at position 2694 where a base Thiamine (T) was substitution with Adenine (A) was observed in 10 isolates (n = 7 from Wagagai isolates, n = 3 from the clinic isolates). Also, a non synonymous polymorphism involving base change from T to A at position 1707 was observed in 2 isolates all from Wagagai samples. This synonymous polymorphism leads to amino acid change from Asperagine (N) to lysine (K) at position 569. Most of the isolates had only one polymorphism; however one isolate had both T2694A and T1707A polymorphisms.

DISCUSSION

The consumption of low doses of artemisinin, in Artemisia tea for a prolonged time may influence resistance to artemisinin its self (World Health Global Malarial Programme: Position statement on the effectiveness of Non-Pharmaceutical Forms of *A. annua* L. against malaria, 2012). Information on Artemesia tea use and its possible association to mutations in artemisinin resistance candidate genes is scarce. This study performed in 2010, genotyped SNPs in the PfATPase6 gene in *P. falciparum* from patients in a Ugandan community who were using Artemisia tea in management of malaria. The two SNPs observed in this study, were also reported by other studies done elsewhere; T1707A in *P. falciparum* from Niger, Zanzibar and Tanzanian (Maman et al., 2009; Dahlstrom et al., 2008). Single nucleotide polymorphism T2694A was the most prevalent. This SNP was also observed in *P.falciparum* isolates from South American (Ferreira et al., 2007; Brasil et al., 2012), and those of the Greater MeKong Subregion (Miao et al., 2013) and was also the most prevalent in the regions. It seems that the two alleles (A or T) are fairly distributed in *P. falciparum* populations (Ferreira et al., 2007), this distribution may not be a result of artemisinin pressure but possibly due to parasite natural evolution. An *in vitro* study of the *P. falciparum* response to artemether or artesunate revealed no association of this mutation with reduced sensitivity to these drugs (Ferreira et al., 2007). Also, Maman et al. (2009), observed no association between T1707A SNP and parasitemia levels in individuals with *P. falciparum* malaria. It is therefore unlikely that these mutations could have been selected by Artemisia tea use or even artemisinin pressure. The T2306A SNP responsible for amino acid substitution from S to N at codon 769 previously reported by Jambou et al. (2005) as a putative marker for artemisinin resistance was not observed in this present study. Current available data indicate that the PfATPase6 gene may not be the one under selection in the case of artemisinin resistance (Miao et al., 2013; Tanabe et al., 2011). Recent studies have identified SNPs in the Kelch and cysteine protease falcipain-2 genes (Ariey et al., 2014) as potential markers for artemisinin resistance.

In conclusion, this study observed two SNPs, however based on other studies done elsewhere, these SNPs where found not to be associated with artemisinin resistance. ACTs still continue to remain efficacious in Uganda (Muhindo et al., 2014), but constant monitoring of practices which may predispose selection of artemisinin resistance *P. falciparum* is imperative. Especially, artemisinin resistance was confirmed in some parts of Asia (Phyo et al., 2012). Future molecular surveillance studies should be focused on Ketlch and cysteine protease falcipain-2 genes as a study by Ariey et al. (2014), associated mutations in these genes, with decreased *in vitro* artemisinin sensitivity and delayed parasite clearance in patients after treatment with artemisinin in Asia.

Some of the limitations of this study were: the few number of isolates studied. The smaller sample size may not have been representative enough for Wagagai community. Also, we did not perform PCR to distinguish between single and multiclonal infections, therefore it was not possible to infer on the clonal diversity of the isolates.

Conflict of Interest

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

ACKNOWLEDGEMENTS

The authors thank Dr Patrick Ogwang for allowing them to use samples he collected in 2010. They are also grateful to Prof. Enock Matovu of Makerere University College of Veterinary Medicine and Biosecurity for allowing them to use his laboratory and reagents to do this work.

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Vol. 8(47), pp. 3815-3820, 19 November, 2014
DOI: 10.5897/AJMR2014.6980
Article Number: B02F4E648992
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Effects of flashing light-emitting diode (LED) of several colors on the growth of the microalga *Isochrysis galbana*

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Received 23 June, 2014; Accepted 13 November, 2014

The growth rate of *Isochrysis galbana* was determined under flashing light conditions (104 µmol $m^{-2}s^{-1}$ light intensity, 10 kHz frequency, and 50% of the duty cycle) of several colors. The most suitable light for the growth of *I. galbana* was blue (dominant wavelength: 470 nm). After six days, the cell density under blue light was 34.0 × 10^5 cells mL^{-1}, and was 1.4, 1.6, 1.8,and 2.2 times higher than those under white, red, white non-flashing, and green light, respectively. The peak wavelengths of white and blue lights are nearly consistent with the absorbance maxima of major pigments, which are chlorophylls and xanthophylls, in *I. galbana*. White and blue light were considered to be most effective for *I. galbana* growth.

Key words: Algal growth, light conditions, light-emitting diode (LED), duty cycles, flash.

INTRODUCTION

Phytoplankton is valuable as a diet for cultured bivalve and shrimp larvae, and the haptophyte *Isochrysis galbana* is widely used in aquaculture (Kaplan et al., 1986; Sukenik and Wahnon, 1991; Saoudi-Helis et al., 1994; Phatarpekar et al., 2000; Sánchez et al., 2000). Mass quantities of this microalga are often cultured indoors to avoid the effects of external factors (Toba and Miyama, 1993a, b; Ueno, 2003). Stable algal cultures are difficult to achieve outdoors because of the influence of weather, low temperature, and contamination by other species. Optimal growth conditions such as nutrients, salinity and temperature have been investigated in previous studies (Kaplan et al., 1986; Toba and Fukayama, 1993a, b). Light conditions are also important factors for controlling indoor microalgae culture.

Although fluorescent lamps are still used as a common light source (Kaplan et al., 1986), light-emitting diodes (LEDs), which have become more affordable, have several advantages (Park and Lee, 2000). For example, LEDs use less electricity than fluorescent lamps and do

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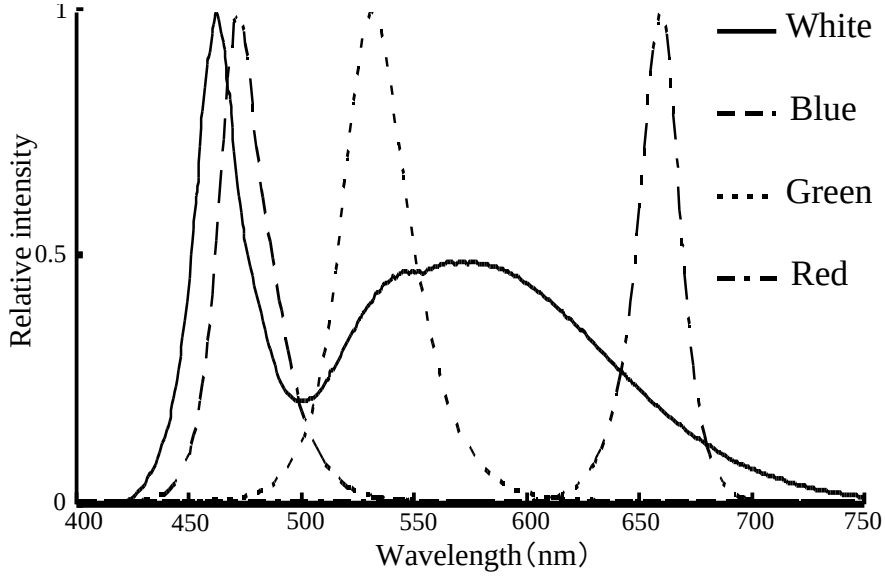

Figure 1. Spectral distributions of the four LED panels used in this study.

not cause the room temperature to rise (Ishikawa and Isowa, 2012). Since the rise in temperature caused by fluorescent lamps decreases the phytoplankton growth rate, LEDs may be a suitable light source for indoor algal culture. This study tested the growth of several phytoplankton species, including *Chaetocerosneo gracile*, *Chlorella pyrenoidosa*, *Heterocapsa circularisquama*, *Nannochloropsis* sp., *Pavlova lutheri*, *Skeletonema costatum* and *Spirulina platensis* under different-colored LEDs (Matthijs et al., 1996; Wang et al., 2007; Oh et al., 2008; Das et al., 2011; Ishikawa and Isowa, 2012). The use of LED lighting has also been reported to be difficult for the growth of the phytoplankton (Miguel et al., 2013). The goal of our study was to determine the optimal light conditions for the mass culture of *I.galbana* using different-colored LEDs as a light source. In our previous study, we investigated suitable flashing light conditions using white light (Yago et al., 2012).

Pigment profiles vary with different classes of phytoplankton (Jeffrey and Wright, 1997). The suitable light colors for phytoplankton growth are known to differ with species (Wang et al., 2007; Ravelonandro et al., 2008). To effectively produce commercially valuable substances in phytoplankton, growth tests using LEDs were conducted (Katsuda et al., 2004; Konishi et al., 2007). In this study, we determined the light colors that produce the highest growth rates of *I. galbana*.

MATERIALS AND METHODS

The basic experimental procedure is the same as in our previous report (Yago et al., 2012).

Experimental organisms and apparatus

I. galbana was obtained from the National Research Institute of Aquaculture, Fisheries Research Agency, Japan, and cultured in f/2 medium (Guillard and Ryther, 1962) at 20°C and 20 µmol photons m^{-2} s^{-1} on a 12:12 h light: dark cycle before experimental use. During experiments, the algae were grown in beakers, each containing 500 mL of f/2 medium. The experiments for each light color were repeated eight times with two beakers for each treatment. The beakers were held in a water bath at 20 ± 0.5°C. Magnetic stirrers kept cells in suspension for uniform illumination. Initial cell densities for each experiment were approximately 2.0 × 10^5 cells mL^{-1}. The experimental and control cultures were grown for 12 days, and cell densities were measured on days 2, 3, 4, 6, 8, 10 and 12.

Effect of the wavelength of flashing light on growth

Flashing or non-flashing light was provided by LED panels (INL-S305x302-WWWW; CCS Inc., Kyoto, Japan). Control cultures received non-flashing white light on a 12:12 h light : dark cycle at 104 µmol m^{-2} s^{-1} of photosynthetically available radiation, which provided a daily irradiance of 4.5 mol m^{-2} d^{-1} (104 µmol m^{-2} s^{-1} × (60×60 ×12) s). Flashing light conditions continued over 24 h, so instantaneous irradiances were reduced to half those of controls to equalize the daily irradiance. We prepared various LEDs with different light colors: white, blue, green and red (Figure 1). The peak wavelengths of each color are 460 and 560 (white), 470 (blue), 525 (green) and 660 nm (red). The flashing light irradiance (52µmol m^{-2} s^{-1}), frequency (10 kHz), and duty cycle (50% light and 50% dark; 52µmol m^{-2} s^{-1}× (60 × 60 ×24) sec) were regulated with a panel control device (ISC-101-4, CCS Inc., Kyoto, Japan). The flashing light pulse profile was rectangular. Irradiance was measured with a LI-COR LI-250 photometer (LI-COR Inc.,Lincoln, NE, USA).

Cell counts and pigment analysis

To compare the growth rates in different treatments and the control cultures, a 10-mL sample was collected from each culture vessel every t days and diluted 20 times with filtered seawater. Microalgae were counted using a Coulter Counter Multisizer II (Beckman Coulter Inc., Brea, CA, USA) with a 100-µm aperture tube.

Chlorophyll*a* (Chl. *a*) concentration was analyzed by the

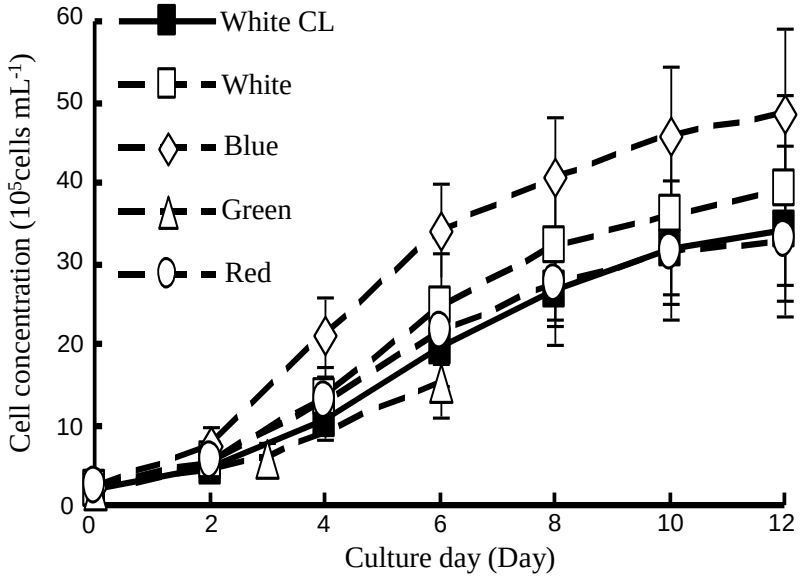

Figure 2. Relationships between culture day and cell concentration under five different light conditions. Points: means ± standard deviations (n = 16). CL: non-flashing light.

fluorometric method (Holm-Hansen et al., 1965). Every 2 days, the 1-mL sample was filtered through a glass fiber filter (47-mm Whatman GF/F filter; Whatman PLC, Springfield Mill, Maidstone, UK). Chl. *a* pigment were extracted from the filter for 24 h in 6 mL of N,N-dimethylformamide (Wako Chemicals, Kyoto, Japan). Chl. *a* concentrations were measured using a Turner Designs fluorometer (Model 10-005R; Sunnyvale, CA, USA).

The major xanthophyll pigments, fucoxanthin (Fuco) and diadinoxanthin (Diadino), were analyzed using high-performance liquid chromatography (HPLC). At the end of the experiment, the 10-mL sample was filtered through a glass fiber filter (25-mm Whatman GF/F filter, Whatman PLC, Springfield Mill). The pigments were extracted from the filter for 24 h in 1 mL methanol (Merck), and the extract was centrifuged at 10000 ×*g* for 10 min. After dilution of the supernatant to 80% with distilled water in an HPLC auto-sampler (Shimadzu, Kyoto, Japan), the pigments were analyzed using the methods of Zapata et al. (2000), with a slight modification whereby a guard column was attached between the injection valve and the analytical column.

Statistical methods

Significant tests of cell density and pigment concentrations were conducted by PSAW ver.17 software (IBM Corporation, Armonk, NY, USA). We used Mann-Whitney's U test of nonparametric measures for a comparison of the average of each group of cell densities, and the Tukey HSD test of parametric measures for a comparison of the average of each group of pigments.

RESULTS AND DISCUSSION

Effects of different colors on the growth of *I. galbana*

I. galbana grew in all experimental plots during the experimental period (Figure 2). On day 6, the cell density decreased in the order, blue light > white light > red light >white non-flashing light > green light (Figure 3). The cell density under blue light was 34.0 × 10^5 cells mL^{-1}, and was 1.4, 1.6, 1.8 and 2.2 times higher than those under white, red, white non-flashing and green light, respectively ($p<0.01$).

Although the suitable light colors for phytoplankton growth differ with species (Wang et al., 2007; Ravelonandro et al., 2008), blue light increased the growth of the diatoms *Biddulphia* sp. (Humphrey, 1983), *Cyclotella nana* (Wallen and Geen, 1971) and *Skeletonema costatum* (Oh et al., 2008), the dinoflagellate *Heterocapsa circularisquama* (Oh et al., 2008), and the green algae *Dunaliella tertiolecta* (Wallen and Geen, 1971) and *Nannochloropsis* sp. (Das et al., 2011). We observed a similar positive effect of blue light on the growth of *I. galbana*.

Major pigments in the haptophyte *I.galbana* include chlorophylls, such as Chl*a*, Chlc_1, Chlc_2 and xanthophylls, such as Fuco and Diadino (Figure 4). These pigments are also major pigments in diatoms (Lavaud et al., 2003).

Photosynthetic pigments are components of the light-harvesting complexes of phytoplankton. Light is trapped in pigments in the photosynthetic system (Matthijs et al., 1996), and it is important for photosynthesis and phytoplankton growth to match the wavelength of light and absorption bands of pigments. The absorbance maxima of Chl.*a* and Diadino are about 430 and 478 nm, respectively (Jeffrey et al., 1997) (Figure 4). In our study, the peak of blue and white light is 470 and 460 nm. The peaks of blue and white lights are nearly consistent with the absorbance maxima of major pigments in *I. galbana*

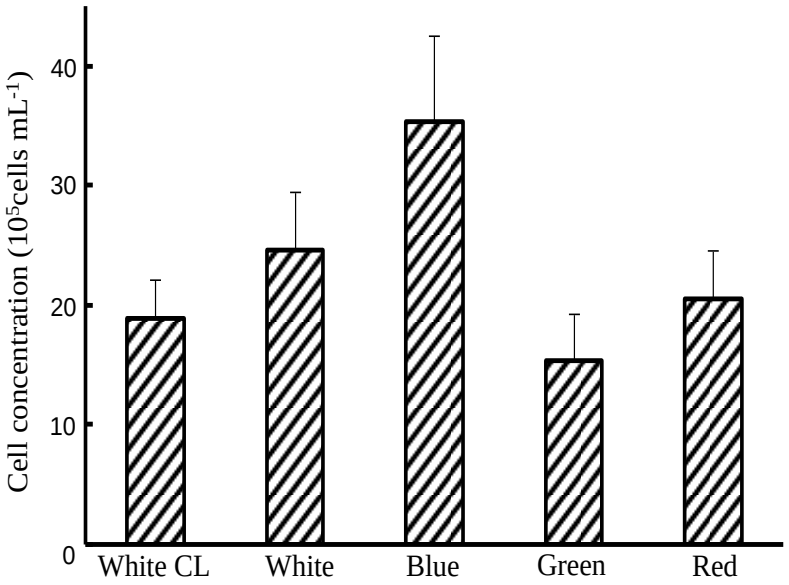

Figure 3. Cell concentrations of five different light conditions on the sixth day of testing. Values are means. Error bars indicate standard deviations (n = 16). CL: non-flashing light. Cell concentration under blue light was significantly higher than all other colors. Cell concentration under green light was significantly lower than blue, white and red light.

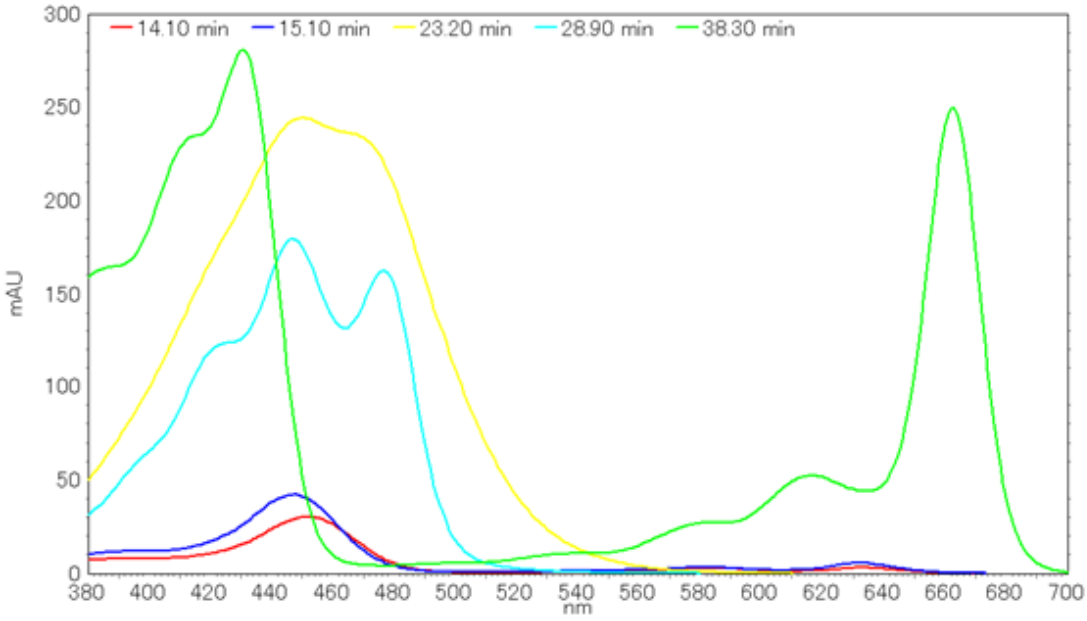

Figure 4. Spectrum of major pigments of *I. galbana*. Red line: chlorophyll c2; retention time for HPLC analysis was 14.1 min. Blue line: chlorophyll c1; retention time was 15.1 min. Yellow line: fucoxanthin; retention time was 23.2 min. Light blue line: diadinoxanthin; retention time was 28.9 min. Green line: chl *a*; retention time was 38.3 min.

and blue and white lights were effective for *I. galbana* photosynthesis. Although blue light has a single absorption peak, white light has a dual peak (Figure 1). In the white light spectrum, the peak area around 560 nm is approximately equal to the peak area around 460 nm. When light intensity is equivalent, the blue light intensity

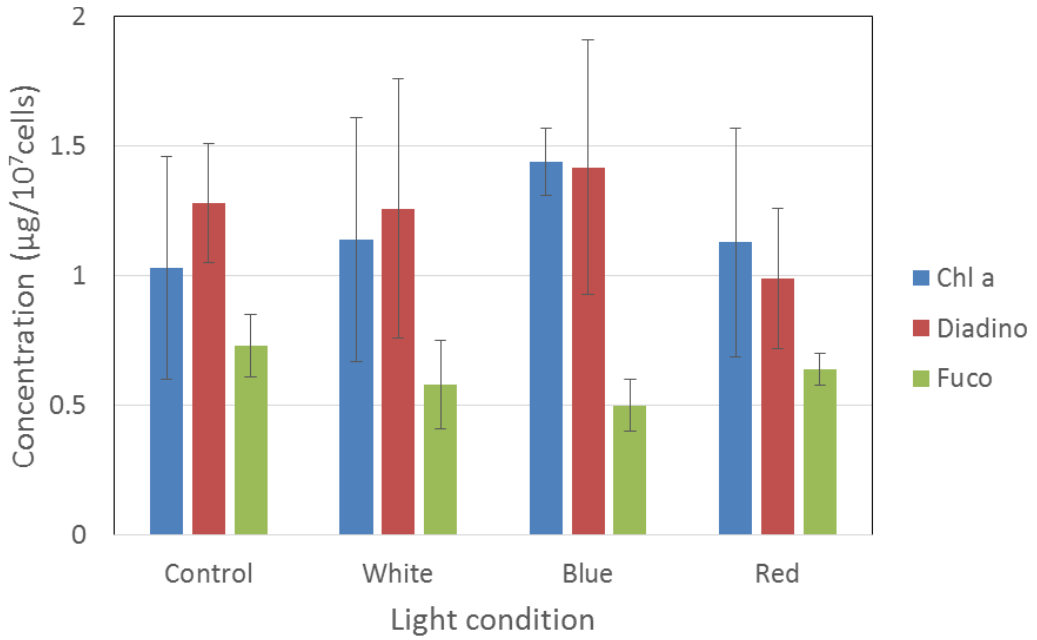

Figure 5. Cell pigment contents (10^{-7}µg $cell^{-1}$) of *I. galbana* under four different light conditions on the 12th day of testing. Chl.*a*: Chlorophyll *a*; Fuco: Fucoxanthin; Diadino: Diadinoxanthin. The values are means±standard deviation (n=4). Control: white continuous light, White: white flashing light, Blue: blue flashing light, Red: red flashing light. As the growth under green light was poor, pigment contents under green light were not measured. Chl*a* concentration under blue light was significantly higher than all other colors. Fuco and diadino were not detected with significant difference by light colors.

around 470 nm was equivalent to about twice that of white light around 460 nm. Therefore, we conjecture that growth under blue light would be higher than that under white light. Chl. *a* has two absorbance maxima (about 435 and 665 nm) (Jeffrey et al., 1997) (Figure 4). The peak of red light is 660 nm, and is nearly consistent with the secondary absorbance maxima of Chl. *a* (about 665 nm). Red light was also effective for photosynthesis of *I. galbana*. Except for cyanobacteria and red algae that possess phycobilin, the green region (500-600 nm) for all plants is of lower absorbance than the blue and red regions (Seki and Naganuma, 1996) (Figure 4). As the peak wavelength of green light (525 nm) does not coincide with the absorbance maxima of major pigments in *I. galbana* (about 430 to 478 and 665 nm), green light is not used for growth by the microalgae (Katsuda et al., 2004).

Effects of different colors on the pigment contents of *I. galbana*

Although Chl *a* concentration under blue light was higher than under other light conditions, there was not a clear difference between Fuco and Diadino concentrations under several light color emissions (Figure 5). In general, Chl. *a* and Fuco concentrations are thought to be higher under low light conditions to increase photosynthesis (Kebede and Ahlgren, 1996; Anning et al., 2000; Macintyre et al., 2002). However, variation in the fucoxanthin concentration was not consistent with Chl*a* concentrations in this study. The concentrations of Diadino and diatoxanthin are thought to be high under high light conditions to enhance a photoprotective effect, which relates to the diadinoxanthin cycle (Anning et al., 2000; Macintyre et al., 2002; Jeffrey and Mantotra, 1997). The variation of Chl. *a*, Fuco and Diadino concentrations under several light colors did not follow a clear trend. The light intensity of the midsummer sun, which can induce light injury is approximately 2,000 µmol m^{-2} s^{-1}, and is 20 times higher than the light intensity used in this study (104 µmol m^{-2} s^{-1}). It is probable that the light intensity in this study was not high enough to induce a photoprotective effect on the pigment profile of *I. galbana*. Therefore, the relationship between light color and each pigment concentration is still unclear.

In our previous study, we determined suitable light conditions for the growth of *I. galbana* under white flashing light (104 µmol $m^{-2}s^{-1}$ light intensity, 10-100 kHz frequencies and 40-80% of the duty cycle) (Yago et al., 2012). Under the same light regime, blue light was found to be particularly suitable for the growth of *I. galbana*. The elongation and leaf formation of the plant are influenced by light quality (Fukuda, 2008). In the future, we would

like to examine the influence of light quality on the growth of phytoplankton from a biochemical perspective.

Conflict of Interest

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

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Vol. 8(47), pp. 3821-3829, 19 November, 2014
DOI: 10.5897/AJMR2014.7146
Article Number: 61B4F6A48994
ISSN 1996-0808

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African Journal of Microbiology Research

Full Length Research Paper

Improved multiplex polymerase chain reaction (PCR) detection of *Bacillus cereus* group and its toxic strains in food and environmental samples

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Received 23 September, 2014; Accepted 18 November, 2014

A multiplex PCR assay for the rapid detection of *Bacillus cereus* group, enterotoxic and emetic strains was developed. A panel of emetic and enterotoxic reference strains, *B. cereus* group members and non-target strains were used for the evaluation of the assay. Verification of PCR results on pure culture and inoculated foods successfully confirmed the specificity of approach for detection of target genes for *B. cereus* group (*groEL*), diarrheal (*cytK*, *nheA*, *hblC*, *entFM*) and emetic strains (*CER*). The sensitivity of approach was satisfying in pure culture as 20 pg of DNA per reaction tube. Artificial contamination of seven different food matrices with distinct bacterial counts revealed a minimum detection limit of 10^3 cfu/g in food samples. The detection limits were improved to approximately 10^1 cfu/g after 7 h enrichment. Natural contamination of rice and kimbab as well as environmental samples (soil, cow feces) was studied. The incidence of *B. cereus* was 63.88 and 38.88% in rice and kimbab, and 84.61 and 69.23% in soil and feces, respectively. To the best of our knowledge, this is the first time that an assay for simultaneous detection of *B. cereus* group, emetic and enterotoxic strains with such a wide range of detection target genes in food and environmental samples has been described.

Key words: *Bacillus cereus* group, multiplex polymerase chain reaction (PCR), enterotoxic strains, emetic strains, food and environmental samples.

INTRODUCTION

Bacillus cereus sensulato, the Gram-positive, rod-shaped, spore-forming opportunistic human pathogen is widely distributed in nature (Bartoszewicz et al., 2008). Therefore, it is an ever-present problem in a broad range of foods (Martínez-Blanch et al., 2010). Cereal products, rice, vegetables, pasta, meat, milk, liquid egg, spices, herbs and texturing agents are among the important food sources of *B. cereus* (Agata et al., 2002; Dierick et al., 2005; Fricker et al., 2007). The *B. cereus sensulato* group includes the species *B. cereus*, *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus weihenstephanensis* (Kim et al., 2012).

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Although *B. cereus* has been reported to be responsible for eye, wound and other systematic infections, it is widely known as a causative agent of gastrointestinal diseases (Kotiranta et al., 2000; Ghelardi et al., 2002). The symptoms of food poisoning resulting from consumption of contaminated food may be either diarrheal and/or emetic type (Sandra et al., 2012). The diarrheal food poisoning is caused by heat-labile enterotoxins produced during vegetative growth of *B. cereus* in the small intestine. Among these protein compounds, non-haemolytic enterotoxin (NHE), enterotoxin FM (ent FM), Haemolysin BL (HBL) and cytotoxin K (cytK) are of the highest importance and therefore often used for the detection of enterotoxic strains (Kim et al., 2012). Symptoms of the diarrheal syndrome include abdominal pain and diarrhea and occur 8-16 h after ingestion of contaminated food which can be occasionally misdiagnosed with *Clostridium perfringens* food poisoning (Park et al., 2009).

The emetic food poisoning is caused by emetic toxin (cereulide), a heat and acid stable small cyclic peptide (dodecadepsipeptide) that induces swelling of mitochondria in Hep-2 cells, respiratory distress, and occasional loss of consciousness possibly leading to coma and ultimately death of individual (Ladeuze et al., 2011). The symptoms usually include nausea, vomiting and stomach pain which occur 1-5 h after food ingestion and can easily be misdiagnosed with *Staphylococcus aureus* food poisoning (Kim et al., 2011). Due to the increasing number of reports of food-borne disease and higher public demand for consumer safety, fast detection methods are required for diagnosis as well as prevention of food contamination (Toh et al., 2004; Yang et al., 2005).

The detection of *B. cereus* toxin producing strains was primarily limited to costly and labor intensive methods such as Hep-2 cell culture vacuolation assay, HPLC-MS analysis and the limited available enterotoxin detection kits. Later on, sperm bioassay for the detection of cereulide-producing strains was developed (Andersson et al., 2004; Buchanan et al., 1994). More recently, molecular diagnostic assays are being developed due to their advantages such as simplicity, high sensitivity, low cost and rapidity (Hyeon et al., 2010), especially after several marker genes of *B. cereus* were identified and characterized. Among the molecular methods, different types of polymerase chain reaction (PCR) have been established as valuable alternatives to the traditional detection methods (Elizaquível et al., 2011).

Ghelardi et al. (2002) used PCR along with other methods for identification of *B. cereus* by toxin genes. Nakano et al. (2004) performed a PCR assay based on a sequence-characterized amplified region marker for the detection of emetic *B. cereus* and Priha et al. (2004) detected *B. cereus* group on cardboard and paper using real-time PCR. In 2006, Ehling-Schulz et al. reported multiplex PCR detection of emetic and enterotoxic strains. Later on, real-time PCR assays were developed by Wehrle et al. (2010) and Martínez-Blanch et al. (2010) for detection and quantification of enterotoxigenic strains, respectively. Also Martínez-Blanch et al. (2010) evaluated a real-time PCR assay for the detection and quantification of *B. cereus* group spores in food and detection and quantification of viable *B. cereus* in food by real-time quantitative PCR (RT-qPCR) (Martínez-Blanch et al., 2011). In our laboratory, we previously developed two multiplex PCR assays, one for the simultaneous detection of *B. cereus* emetic and enterotoxic strains (Kim et al., 2012) and one for the detection of *B. cereus* emetic strains (Kim et al., 2013).

Despite the advantages of the above mentioned methods, none of them could simultaneously detect and identify *B. cereus sensulato* group non-toxic members, enterotoxic and emetic strains together. Some of them detected *B. cereus* group, some the enterotoxic and/or emetic strains and finally, some detected *B. cereus* group with either emetic or enterotoxic strains. Thus, the aims of this study were to 1) develop an efficient multiplex PCR assay to simultaneously detect *B. cereus* group, its enterotoxic and emetic strains and 2) to evaluate the assay in a wide variety of foods to ensure practical suitability. An advantage of this method would be its ability to detect members of *B. mycoides* and/or *B. thuringiensis* which may not produce any of the known toxins resulting in foodborne illness (Priha et al., 2004; Yang et al., 2005). Hence, this approach could be a useful tool not only for the detection of *B. cereus* group toxic strains but also for the large scale monitoring, genotyping and preliminary studies in search of *B. cereus sensulato* in foods and other samples.

MATERIALS AND METHODS

Bacterial strains

A total of 35 reference strains including 11 emetic *B. cereus* reference strains, 7 enterotoxic *B. cereus* reference strains, 5 members of *B. cereus* group and finally 12 other foodborne pathogens were used in the study (Table 1). All strains were obtained from the Department of Food Science and Biotechnology, Kangwon National University, South Korea.

DNA isolation

All strains were grown on tryptic soy agar (TSA; Difco, Detroit, MI, U.S.A.) plates at 35°C for 24 h. A single colony was inoculated into Luria-Bertani (LB broth; Difco) broth and incubated at 35°C for 8 h. One milliliter of the pure culture was centrifuged at 5000× g for 10 min at 4°C and the DNA was extracted using a DNeasy Tissue Kit (Qiagen AB, Uppsala, Sweden) according to the manufacturer's instructions. The purified DNA was recovered in 100 µL of sterilized distilled water and DNA concentration was determined by using a NanoDrop ND-2000 UV/VIS spectrophotometer (NanoDrop Technologies) at 260 nm. The purified DNA was diluted to reach the concentration of approximately 15-20 ng/µL and stored at -20°C for the following experiments.

Primers

Table 2 shows the primers used in this study. The four primer pairs

Table 1. List of reference strains used for the evaluation of multiplex PCR assay.

Emetic reference strains	Enterotoxic reference strains	*B. cereus* group members	Non-target strains
B. cereus F4810/72	*B. cereus* ATCC13061	*B. thuringiensis*KCTC1508	*Escherichia coli* KCCM32396
B. cereus JNHE36	*B. cereus* ATCC12480	*B. thuringiensis*824	*Escherichia coli* O157:H7 ATCC 43895
B. cereus JNHE78	*B. cereus* KCTC1013	*B. mycoides*KCTC 3453	*Salmonella* typhimuriumATCC14028
B. cereus KUGH164	*B. cereus* KCTC1014	*B. weihenstephanensis*KACC12001	*Listeria monocytogenes*ATCC19119
B. cereus KNIHuls1	*B. cereus* KCTC1092	*B. pseudomycoides*KACC12098	*Listeria monocytogenes*ATCC19115
B. cereus KNIHuls3	*B. cereus* KCTC1094		*Staphylococcus aureus* ATCC12500
B. cereus KNIHuls4	*B. cereus* KCTC1526		*Staphylococcus aureus* ATCC27729
B. cereus KNIHuls5			*Clustridiumperfringens*KCTC5101
B. cereus KNIHuls7			*B. subtilis* KCCM11316
B. cereus KNIHuls8			*B. subtilis* KCTC3135
B. cereus KFDA250			*Sseudomonasputida*KCCM35479
			*Escherichia coli*ATCC3565

Table 2. Primers used in this study for the multiplex PCR assay.

Target gene	Sequence (5´→ 3´)	Product size (bp)	Design source	Reference
cytK	TGCTAGTAGTGCTGTAACTC CGTTGTTTCCAACCCAGT	881	DQ019311	Kim et al., 2012
nheA	GGAGGGGCAAACAGAAGTGAA CGAAGAGCTGCTTCTCTCGT	750	DQ019312	Kim et al., 2012
CER	GCGTACCAAATCACCCGTTC TGCAGGTGGCACACTTGTTA	546	AY576054	Kim et al., 2012
hblC	CGCAACGACAAATCAATGAA ATTGCTTCACGAGCTGCTTT	421	AY786407	Kim et al., 2012
entFM	AGGCCCAGCTACATACAACG CCACTGCAGTCAAAACCAGC	327	AY789084	Kim et al., 2012
groEL	AGCTATGATTCGTGAAGGT AAGTAATAACGCCGTCGT	236	AB077143	Kim et al., 2013

for the detection of enterotoxin genes (*cytK, nheA, hblC, entFM*) and *CER* primers for the detection of emetic strains were previously used in a multiplex PCR assay to detect enterotoxic and emetic strains simultaneously (Kim et al., 2012). The *groEL* primer pair targeting *B. cereus sensulato* group using gene sequences encoding molecular chaperonins was also successfully evaluated in our previous studies (Kim et al., 2013) as well as others (Chang et al., 2003) for the detection of *B. cereus* group. All primers were commercially produced by Bioneer (Daejeon, South Korea) using *AccuOligo*® technology (http://eng.bioneer.com/products/Oligo/CustomOligonucleotides-technical.aspx).

Optimization of multiplex PCR assay

One emetic reference strain (F4810/72) and one enterotoxic reference strain (ATCC12480) were used for optimization of the multiplex PCR assay. In brief, ability of primers to be used in a multiplex assay was checked and primer concentrations were optimized in singleplex, duplex and multiplex PCRas well as optimum PCR conditions by trial and error. All PCR assays were conducted using Mygenie32 Thermal Block (Bioneer) thermal cycler. PCR products were checked on 2% agarose gel (Sigma-Aldrich; St. Louis, MO, USA) at 100 V for 30 min in × 0.5 Tris-borate EDTA (TBE) buffer using electrophoresis machine (Mupid-exU, Mupid, Tokyo, Japan). The gels were stained with SafeView™ (ABM, Richmond, BC, Canada) and visualized using a UV transilluminator (Gel Doc 2000; Bio-Rad, Hercules, CA, USA). A 100-bp DNA ladder (Solgent, Daejeon, South Korea) was used as molecular weight marker.

Evaluation of multiplex PCR assay

The specificity of the primers as well as their ability to perform in a multiplex PCR assay was confirmed by assessing 35 bacterial strains. These included a panel of 11 emetic reference strains, 7 enterotoxic reference strains and 5 strains of *B. cereus* group members. Furthermore 12 non-target Gram-positive and Gram-negative bacterial species were tested (Table 1). Gene profiles of the reference strains resulted in this study were compared with

previous data to confirm the specificity of the developed multiplex PCR assay.The specificity of *CER* primer was evaluated using emetic reference strains and non-target strains and enterotoxin gene primers were evaluated using the enterotoxic reference strains as well as non-target strains. Finally, the specificity of *groEL* primer set was evaluated using the emetic and enterotoxic strains as well as *B. cereus sensulato* group members.

Detection limit in pure culture

Detection limit of the developed assay in pure culture was determined using 4 enterotoxic (ATCC13061, ATCC12480, KCTC1013, KCTC1094) and one emetic (F4810/72) *B. cereus* reference strain. After preparing tenfold dilution series of pure DNA from each strain ranging from 20 ng to 200 fg, each dilution was subjected to multiplex PCR assay.

Artificially inoculated food assays

Baby cereal, kimbab (Korean food containing rice and other ingredients rolled in seaweed), pasteurized milk, pasta, rice, sunflower oil and tteok (Korean rice cake made with glutinous rice flour) were purchased from local food stores in Chuncheon, South Korea. If necessary, food samples were autoclaved and tested for the absence of any naturally occurring contamination with *B. cereus* by the standard reference culture methods (Fricker et al., 2007). In brief, 25 g of sample was homogenized in a filter stomacher bag (Nasco Whirl-Pak, Janesville,WI) using a Seward stomacher (400 Circulator,Seward, London, UK) with 225 mL of brain heart broth (BHIG; Merck, Darmstadt, Germany) supplemented with 0.1% glucose. The homogenate was incubated at 37°C for 24 h without shaking. Subsequently, 100 µL of each dilution was spread onto mannitol-eggyolk-polymixin agar (MYP; Difco) to check presence of *B. cereus* cells. Samples confirmed for the absence of *B. cereus* were used for artificial contamination.

For inoculation, one emetic (F4810/72) and three enterotoxic reference strains (ATCC12480, ATCC13061 and KCTC1014) were separately seeded into 25 g of food and homogenized for 2 min with 225 mL of 0.1% buffered peptone water (BPW; Difco) or tryptic soy broth (TSB; Difco) as mentioned above resulting in a final *B. cereus* cell concentration of approximately 1.8×10^6, 4.2×10^6, and 2.1×10^6 and 2.7×10^6 cfu/g of *B. cereus* F4810/72, ATCC 13061, ATCC12480 and KCTC 1014, respectively. Serial dilutions covering a range of 10^6 to 10^1 cfu/g were prepared and DNA was immediately extracted from 1 mL of BPW diluted homogenates using NucleoSpin food kit (Macherey-Nagel, Germany) according to the manufacturer's guidelines. TSB diluted homogenates were incubated at 37°C for 7 h with 200 rpm agitation and DNA was extracted as mentioned above. DNA extracts of each dilution were used for multiplex PCR assay while in parallel 0.1 and 1 mL of each dilution was spread plated onto MYP agar to compare the developed approach efficiency with conventional culture method.

Analysis of naturally contaminated food

Rice and kimbab (36 samples, each) were purchased from local markets, wholesales and restaurants in Kangwon-do province, South Korea and immediately transferred to laboratory to test the natural contamination. In brief, 25 g of each sample was homogenized for 2 min with 225 mL of 0.1% BPW (Difco). 1 mL of the homogenate was used for DNA extraction as mentioned above and subjected to the developed multiplex PCR assay. All experiments, including PCR and conventional culture methods were conducted in duplicate.

Analysis of soil and feces

Soil samples (13 samples) were collected in Kangwon National University campus. Samples were taken with a sterilized spoon from a depth of 10 to 20 cm into a stomacher bag. All samples were treated on the same day of collection. Cow feces (13 samples) were collected into sterile containers from cattle at Kangwon National University farm from individual animals and analyzed within the same day.

Briefly, 10 g of each sample was homogenized for 5 min with 90 mL of 0.1% BPW or TSB as mentioned above. The sample was left to settle for 2 min and 1 mL of the liquid phase was used for DNA extraction using the DNeasy Tissue Kit (Qiagen) as mentioned above. In parallel, 0.1 mL aliquots were used for spread plating on MYP agar (Difco).

RESULTS

Optimization of the multiplex PCR assay

The optimum PCR mixture (25 µL) contained 800 nM of *cytK* and *nheA* primers, 500 nM of *CER*, *hblC* and *groEL* primers and 400 nM of *entFM* primers, approximately 15-20 ng of DNA template, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 40 mM KCl, 250 µM dNTP mixture and 1 U Taq polymerase (Takara Taq™, Otsu, Japan). The optimum amplification conditions were as follows: initial denaturation at 95°C for 10 min, 35 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 1 min and a final extension at 72°C for 5 min.

Evaluation of multiplex PCR assay

Gene profiles of the *B. cereus* reference strains, obtained from the multiplex PCR assay were in agreement with the previous reports, confirming the specificity of the approach (Table 3). Gel electrophoresis of the multiplex PCR results on the 18 *B. cereus* reference strains used in this study confirmed the presence of expected 881, 750, 546, 421, 327 and 236 bp amplicons for *cytK*, *nheA*, *CER*, *hblC*, *entFM* and *groEL*. Also no amplicons were observed for the non-target strains. Figure 1 shows the agarose gel electrophoresis for 6 of the reference strains used in this study. The ability of *groEL* primer to specifically detect *B. cereus* group members was further confirmed by using 5 members of *B. cereus* group members in addition to the reference strains. The *groEL* primer successfully detected all the 5 *B. cereus* group member strains.

Detection limits in pure culture and artificially contaminated foods

The results obtained from multiplex PCR assay of 5 *B. cereus* reference strains used for sensitivity study in pure culture were positive for all strains up to 20 pg of DNA/reaction tube which corresponds to approximately 3×10^3 cfu/mL of bacterial cells.

Figure 2 shows the detection of the four reference strains

Table 3. Gene profiles of emetic and enterotoxic reference strains of *B. cereus* using multiplex PCR assay.

Strain	Type	Multiplex PCR assay					
		CER	***groEL***	***Cyt K***	***entFM***	***hblC***	***nheA***
B. cereus ATCC13061	Enterotoxic	-	+	-	+	-	+
B. cereus ATCC12480	Enterotoxic	-	+	+	+	+	+
B. cereus KCTC1013	Enterotoxic	-	+	+	+	+	+
B. cereus KCTC1014	Enterotoxic	-	+	-	+	-	+
B. cereus KCTC1092	Enterotoxic	-	+	+	+	+	+
B. cereus KCTC1094	Enterotoxic	-	+	+	+	+	+
B. cereus KCTC1526	Enterotoxic	-	+	+	+	+	+
B. cereus F4810/72	Emetic	+	+	-	+	-	+
B. cereus JNHE36	Emetic	+	+	+	+	+	+
B. cereus JNHE78	Emetic	+	+	-	-	-	+
B. cereus KUGH164	Emetic	+	+	-	-	-	+
B. cereus KNIHuls1	Emetic	+	+	-	+	-	+
B. cereus KNIHuls3	Emetic	+	+	-	+	-	+
B. cereus KNIHuls4	Emetic	+	+	-	+	-	+
B. cereus KNIHuls5	Emetic	+	+	-	+	-	+
B. cereus KNIH7uls7	Emetic	+	+	-	+	-	+
B. cereus KNIHuls8	Emetic	+	+	-	+	-	+
B. cereus KFDA250	Emetic	+	+	-	+	-	+

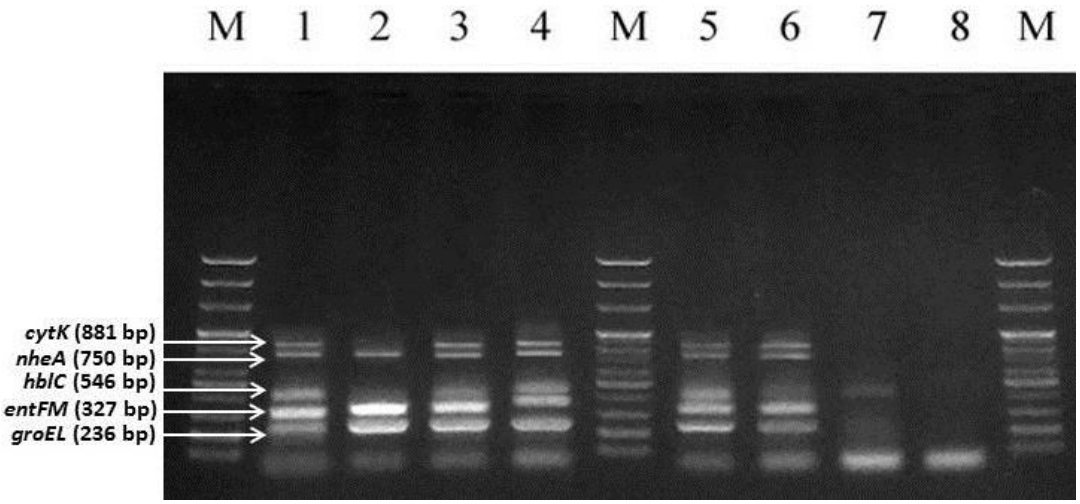

Figure 1. Gel electrophoresis results of 6 enterotoxic reference strains and 1 non-target strain. M, 100-bp DNA size marker; Lane 1, *B. cereus* KCTC1013; Lane 2, *B. cereus* KCTC1014; Lane 3, *B. cereus* KCTC1092; Lane 4, *B. cereus* ATCC12480; Lane 5, *B. cereus* KCTC1526; Lane 6, *B. cereus* KCTC1508; Lane 7, *Pseudomonas putida* KCCM35479; Lane 8, no template control (NTC).

in artificially inoculated food (kimbab). Detection limits of the multiplex PCR assay in food were evaluated using 7 foods (Baby cereal, kimbab, pasteurized milk, pasta, rice, sunflower oil and tteok). Results showed detection limits of 1.8×10^3, 4.2×10^3, 2.1×10^3 and 2.7×10^3 cfu/mL for *B. cereus* F4810/72, ATCC 13061 ATCC12480 and KCTC1014, respectively, for all foods without enrichment. These limits were similar or tenfold higher than the spread cultures performed in parallel. The sensitivity of the approach improved to approximately 10^1 cfu/mL after enrichment (37°C for 7 h with 200 rpm agitation in TSB).

Analysis of naturally contaminated food

Table 4 shows the summary of toxin gene profiles obtained

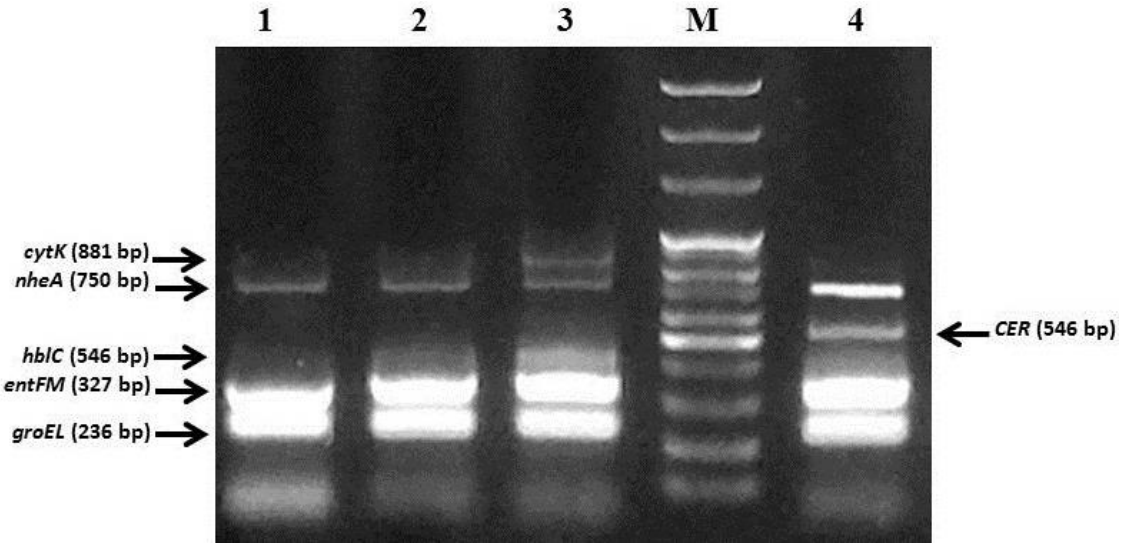

Figure 2. Gel electrophoresis results of 4 *B. cereus* reference strains detection by multiplex PCR approach in artificially inoculated food (kimbab). M, 100-bp DNA size marker; Lane 1, *B. cereus* ATCC 13061; Lane 2, *B. cereus* KCTC 1014; Lane 3, *B. cereus* ATCC 12480; Lane 4, *B. cereus* F4810/72.

Table 4. Summary of toxin genes distribution in positive samples of naturally contaminated rice, kimbab, soil and feces.

Toxin gene	Kimbab (n = 12)	Rice (n = 23)	Soil (n = 11)	Feces (n = 9)
cytK	4 (33.33%)	12 (52.17%)	6 (54.54%)	6 (66.66%)
nheA	11 (91.66%)	20 (86.95%)	11 (100%)	8 (88.88%)
hblC	7 (58.33%)	16 (69.56%)	7 (63.63%)	5 (55.55%)
entFM	9 (75%)	18 (78.26%)	7 (63.63%)	5 (55.55%)
CER	ND	2 (8.69%)	ND	ND

ND: not detected.

in naturally contaminated foods, soil and feces. Among the 36 rice samples analyzed, contamination with *B. cereus* was detected in 23 of them (63.88 %). Among the contaminated samples only two (8.69%) were positive for *CER* primer meaning the presence of emetic strains. Other toxin genes showed different frequencies with *NHE* being the most frequent gene detected (86.95% of positive samples). In kimbab, 12 out of the total 36 (33.33%) samples analyzed were contaminated by *B. cereus* group while no contamination by emetic strains was detected and *NHE* showed the highest frequency as well as in rice while the frequency orders of other toxingenes were different from rice.

Analysis of *B. cereus* presence in soil and feces

In soil samples (Table 4), 11 out of 13 (84.61%) were found positive for the presence of *B. cereus* group. This result was in comparison with direct plating on MYP agar. In case of feces samples, 9 out of 13 (69.23%) were found positive for *B. cereus* presence while direct spread plating was positive for 7 samples (53.84%). This might be due to the lower detection limits or that *B. cereus* cells in one samples did not grow by culturing due to some reasons (death or incapability to adjust to the new medium) but still they were detected by the multiplex PCR approach.

DISCUSSION

B. cereus has been increasingly involved in several foodborne outbreaks (Wehrle et al., 2010). Therefore, there has been increasing interest in the development of appropriate methods for its detection in food. Recently, PCR procedures have been found suitable for pathogen detection in food products since they are rapid and

simple to use (Elizaquível et al., 2011). These advantages were further extended by the application of multiplex PCR allowing amplification of all sequences of interest simultaneously in a "multiple" reaction (Wu et al., 2007). Therefore, in this study four primer pairs (*cytK, nheA, hblC* and *entFM*) for the detection of four commonly present enterotoxin genes in *B. cereus* group as well as *CER* primer for the detection of emetic strains were used, resulting in a wide range of detection to maximize the chance for the detection of *B. cereus* group in food and environmental samples since usually each enteropathogenic strain contains at least one of these genes.

Based on the literature (Priha et al., 2004; Yang et al., 2005), it is possible for some of the *B. cereus* group members, especially *B. thuringiensis* and *B. mycoides* to not contain any of the toxin genes. This information becomes more important knowing that a big portion of *B. cereus* strains isolated from environmental as well as food samples consists of *B. thuringiensis* and *B. mycoides* (Rosenquist et al., 2005). Wehrle et al. (2010) developed a multiplexreal-time PCR based on SYBR green I for the detection of enteropathogenic *B. cereus*. This approach enabled fast and reliable detection of enteropathogenic *B. cereus.* However, it lacked the ability to detect enterotoxin FM gene (*entFM*), which prevalence studies have detected in most *B. cereus* outbreak-associated strains (Kim et al., 2009; Tran et al., 2010).

The first approach to detect and quantify viable *B. cereus* in food was developed by Martínez-Blanch et al. (2011). This approach detected viable *B. cereus* group cells by targeting the phosphatidylcholine-specific phospholipase C (*pc-plc*) mRNA. However, it only detected *B. cereus* group and could not differentiate between toxigenic and non-toxigenic strains. In the present study, we added the *groEL* primer set to the multiplex approach. Therefore, the present approach is able to detect and differentiate *B. cereus* toxigenic group members and also the strains that do not contain any of the known toxin genes.

Usually, foods with *B. cereus* contamination levels below 10^3 cfu/g are considered safe for the consumers (Martínez-Blanch et al., 2011). However, different legal limits have occasionally been set in some countries (Häggblom et al., 2002). In order to further confirm the practical applicability of the approach, 7 foods prone to *B. cereus* contamination were artificially inoculated and analyzed. The developed multiplex PCR approach showed a detection limit of approximately 3×10^2 to 10^3 cfu/mL in pure culture. This result was similar to our previous studies (Kim et al., 2012, 2013). However, interestingly, the detection limits in inoculated foods were 1.8×10^3 and 2.1×10^3 cfu/mL for *B. cereus* F4810/72 and ATCC12480, respectively, for all foods without enrichment which did not always show a tenfold lower limit as compared to the pure culture. The improved sensitivity of the approach in food as compared to the previous studies (Alarcon et al., 2005) might be due to the different DNA extraction method used in this study. As it is well known, foods have a complex matrix containing nucleases, cations, proteases, fatty acids and other PCR inhibitors. Therefore, application of an efficient DNA extraction/purification method may eventually improve the detection limit of PCR approach. Hence, the sensitivity (10^3 cfu/g) of the approach was enough to detect minimum levels of contamination considered as legal limit in food samples, more sensitive than the assay developed by Martı´nez-Blanchet al. (2011) and similar to some other previous results (Wehrle et al., 2010; Kim et al., 2012, 2013) and more sensitive than the work of Nakano et al. (2004), in which the developed approach was not sensitive enough for the direct detection without a 7 h enrichment step. However, typical cell counts in food samples connected to food poisoning are usually much higher in the range of 10^5 to 10^8 cfu/g of *B. cereus* (Fricker et al., 2007). The detection limit was improved to approximately 10^1 cfu/gof *B. cereus* after enrichment. Nakano et al. (2004) also reported the efficiency of a similar enrichment. In this study, TSB medium was used instead of BHI and 37°C instead of 35°C. On the other hand, the conventional culture method not only takes at least 24 h to get the results but also practically is not as sensitive as expected. Although, using 1 mL aliquots may improve sensitivity but such a high volume makes handling of plates harder, needs more time and finally is hard to perform.

Foods containing rice are the main source of *B. cereus* contamination. Cooked or fried rice is involved in approximately 95% of *B. cereus* emetic food poisonings. Despite the lower prevalence of emetic food poisoning as compared to the enterotoxic syndromes, it is still of quite importance as it also can have negative health effects and may occasionally even lead to liver failure or death (Ladeuze et al., 2011; Martinez-Blanch et al., 2009). Therefore, rice and kimbab, as two main foods in the daily diet of Koreans were selected to evaluate the developed approach in naturally contaminated foods and also to perform a preliminary test on the level of *B. cereus* contamination in these foods in Chuncheon city/Kangwon-do province.

Only 2 rice samples (8.69% of total contaminated samples) were positive for *CER* primer while no emetic strains were detected in kimbab. These results are in agreement with the works of Ehling-Schulzet al. (2006), reporting a low incidence for emetic strains and Park et al. (2009), reporting a low incidence for emetic strains in cereals. In general, 63.88 and 33.33% of rice and kimbab samples, respectively, were contaminated by *B. cereus*. Kim et al. (2009) reported an average contamination of 41.7% by *B. cereus* without enrichment in rice samples in South Korea. In the same study, contamination was detected in 95% of the samples after enrichment. In another study, a 40% contamination level was reported (Jang et al., 2006). In kimbab, Cho et al. (2008) reported a 43.3% *B. cereus* contamination level. An important point to be mentioned here might be the application of

only one target gene in above mentioned studies for the detection of *B. cereus* contamination in food samples. Therefore, the reported incidences might be lower than the actual numbers since *B. cereus* strains have a high diversity especially in case of toxigenic profiles. A study performed in Malaysia reported contamination levels of 100, 76.2, 70.4 and 50% in different types of raw rice (Sandra et al., 2012). The results obtained in this study show that there is a need for further study on the contamination levels of products such as rice and kimbab and also to be more careful about performing appropriate actions such as good washing of raw rice prior to cooking, keeping hygiene and preventing cross contamination during handling and storage of cooked products in appropriate temperatures. Also following good agricultural practices (GAP) is necessary for the production of rice as well as similar products since results of the soil and cow feces analysis revealed a high incidence of *B. cereus* in these sources which can easily be transferred to the crop on the field or during the process chain.

In conclusion, the developed multiplex PCR approach meets requirements as a useful tool for the rapid detection of *B. cereus* group in food and environmental samples, as well as identification of enteropathogenic strains. Future studies will have to reveal the capability of the present approach to be used as a combined assay such as with propidiummonoazide DNA intercalating dye for the rapid detection of viable strains, for the toxigenic profiling of *B. cereus* or in combination with most probable number to obtain a MPN-PCR method with quantitative and qualitative detection capability.

Conflict of Interest

Authors have no conflict of interest.

ACKNOWLEDGEMENT

This research was supported by Bio-industry Technology Development Program (112137-3) Ministry of Agriculture, Food and Rural Affairs.

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Vol. 8(47), pp. 3830-3836, 19 November, 2014
DOI: 10.5897/AJMR2013.5871
Article Number: 23A0B3F48996
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Races and virulence of *Puccinia graminis* f. sp. *tritici* in some regions of Iran

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Received 24 May, 2013; Accepted 13 November, 2014

Stem rust represents the major threat to wheat production in Iran. It has been present for many years and epidemics occur in different regions on both winter and spring wheat. The objective of this study was to characterize races of *Puccinia graminis* f. sp. *tritici* present in Khuzestan and Lorstan provinces of Iran in 2012. Using the international system of nomenclature for *P. graminis* f. sp. *tritici*, 20 races were identified. A total of 20 races were identified from 30 isolates, which included the most prevalent races TTKSK and TTJQC with a frequency of 36.4 and 33.3%, respectively. The second most frequent and dominant race was KTTSK with a frequency of 18.2%. The resistance genes *Sr24* and *Sr36* were found to confer resistance to most of the races prevalent from two provinces while genes *Sr5, Sr9e, Sr7b, Sr6, Sr8a, Sr9g, Sr30, Sr9a, Sr9d, Sr10* and *SrMcn* were ineffective against most of the races detected. Among these genes, resistance genes, *Sr11*, *Sr38* and *SrTmp* were found as low infection types (more effective resistance genes) against most of the races detected. The wide virulence diversity that was found in this study across regions and over time will undoubtedly render the task of breeding durably resistant materials more difficult.

Key words: Pathogen races, resistance genes, wheat stem rust.

INTRODUCTION

Stem rust caused by *Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn., is a highly damaging disease of wheat that primarily occurs in warm weather and can cause great damage to susceptible wheat varieties. The rust diseases of wheat have historically been one of the major biotic production constraints both in Asia and the rest of the world. There are more than 3000 rust species in the world (Ershad, 1995; Abbasi et al., 2005), three of which are pathogenic on wheat. These include *P. graminis* f. sp. *tritici* (causal agent of stem rust), *Puccinia striiformis* f. sp. *tritici* (causal agent of stripe rust) and *Puccinia triticina* (causal agent of leaf rust). In most wheat producing areas, yield losses are caused by *P. graminis* Pers. f. sp. *tritici* Eriks. & E. Henn. Studies on stem rust have been undertaken since the 1760s throughout the world, and there are records of stem rust in Iran dating back to 1946 (Esfandiari, 1947). Although *P. graminis* has been observed on local wheat cultivars (Sharif et al., 1970), there were no significant outbreaks of the disease since the last outbreaks in 1975-76 that occurred in Caspian

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Sea and southern parts of the country (Bamdadian and Torabi, 1978).

According to Bamdadian and Torabi (1999) stem rust was historically a major problem in Iran. More than 50 numerically catalogued resistance (*Sr*) genes confer resistance to the various races of the stem rust pathogen. However, virulence for a large proportion of these genes is now common. Although *P. graminis* has been observed occasionally on local wheat cultivars, there were no significant outbreaks of the disease since the last epidemics occurred in the southern parts of the country on Iran during 1975-78 (Bamdadian and Torabi, 1978). New stem rust pathotypes identified in Uganda in 1999 (Ug99) has altered this quiet period of stem rust. Ug99 have defeated an important number of effective resistance genes including those that were used in several breeding programs.

Since first reported in 1999 (Pretorius et al., 2000), TTKSK and its variants have been found throughout eastern and southern Africa (Jin et al., 2008; Singh et al., 2011; Visser et al., 2010; Wanyera et al., 2006; Wolday et al., 2011) and in 2007 Ug99 was detected in the southwest of Iran (Nazari et al., 2009). This is of paramount importance in developing wheat cultivars with durable stem rust resistance. In addition, virulence surveys are important for studying the evolution of new races and forecasting the virulence shifts in a population. The objective of this study, were to analyze the genetic relationship and spatial distribution of physiologic races of *P. graminis* f. sp. *tritici* in the major wheat growing areas in Khuzestan and Lorstan provinces of Iran.

MATERIALS AND METHODS

In this study, field surveys were conducted in the southeast (Ahvaz, Dezful, Behbahan, Shushtar, Shadegan, Izeh and western (Khorramabad, Aligoodarz, Dorood, Borujerd, Koohdasht) regions of Iran in 2012 cropping seasons to collect samples of wheat stem rust. Samples of infected stems were collected at 5-10 km interval from wheat fields. Seedlings of the universally rust susceptible variety “Morocco” which does not carry any known stem rust resistance genes were raised in suitable 8 cm diameter pots. Leaves of seven-day-old seedlings or seedlings with fully expanded primary leaves and second leaves, were rubbed gently with clean moistened fingers. Greenhouse inoculations were done using the methods and procedures developed by Stakman et al. (1962). Spores from the stem rust infected sample were scraped off with scalpels on to a watch glass and suspended in distilled water to make rust spore suspension, which was rubbed on the seedlings of Morocco. The plants were then moistened with fine droplets of distilled water produced with an atomizer and placed in an incubation chamber for 18 h dark at 18 to 22°C followed by exposure to light for 3 to 4 h to provide condition for infection and seedlings were allowed to dry their dew for about 1 to 2 h. The seedlings were transferred from the dew chamber to glass compartments in the greenhouse where conditions were regulated at 12 h photoperiod, at temperature of 18 to 25°C and relative humidity (RH) of 60 to 70%. The remaining rust spore samples were kept in the refrigerator at 4°C and were used for samples which failed to produce infection on the universally susceptible variety in the greenhouse. After seven to ten days, leaves containing a single fleck that produced a single pustule, were selected from the base of the leaves. The remaining seedlings within the pots were removed using scissors.

After two weeks, spores from each pustule were collected using a power-operated vacuum aspirator and stored separately in gelatine capsules. A suspension, prepared by mixing urediospores with lightweight mineral oil (Soltrol 170), was used for inoculating seven-day-old seedlings of the susceptible variety 'Morocco' for multiplication purposes. This was done for each of the single pustules on separate pots. Immediately after inoculation, the seedlings were placed in a humid chamber at 18 to 22°C for 18 h in the dark and for 3 to 4 h in the light, after which they were transferred to a greenhouse. About 14 to 15 days after inoculation, the spores of each single pustule were collected in separate test tubes and stored at 4°C until they were inoculated on the standard differential sets.

Five seeds of the twenty wheat stem rust differentials with known resistance genes (*Sr5*, *Sr6*, *Sr7b*, *Sr8a*, *Sr9a*, *Sr9b*, *Sr9d*, *Sr9e*, *Sr9g*, *Sr10*, *Sr11*, *Sr31*, *Sr17*, *Sr21*, *Sr30*, *Sr36*, *Sr38*, *Sr24*, *SrTmp* and *SrMcN*) and one susceptible variety Morocco were separately grown in 3 cm diameter pots in the greenhouse (Table 1). The single pustule-derived spores were suspended in distilled water and inoculated onto seven-day-old seedlings using atomizers and/or an air pump. After inoculation, plants were moistened with fine droplets of distilled water produced with an atomizer and placed in an incubation chamber at 18 to 22°C for 18 h in the dark and 3 to 4 h in the light. The seedlings were allowed to remove their dew for about 1 to 2 h in a dew chamber. Upon removal from the dew chamber, plants were placed in separate glass compartments in a greenhouse to avoid contamination.

Stem rust infection types (ITs) were scored 14 days after inoculation using the 0 to 4 scale of Stakman et al. (1962). Infection types were grouped in to two, where, low (resistant) = (0, 0; (fleck), 1, 1+, 2 and 2+) and high (susceptible) = (3-, 3+ and 4). Race designation was done by grouping the differential hosts into five subsets in the following order: (i) *Sr5*, *Sr21*, *Sr9e* and *Sr7b*; (ii) *Sr11*, *Sr6*, *Sr8a*, and *Sr9g*; (iii) *Sr36*, *Sr9b*, *Sr30* and *Sr17*; (iv) *Sr9a*, *Sr9d*, *Sr10*, *SrTmp*; and (v) *Sr24*, *Sr31*, *Sr38* and *SrMcN* (Table 1). Each isolate was assigned a five letter race code based on its reaction on the differential hosts (Roelfs and Martens, 1988; Jin et al., 2008).

RESULTS

Virulence structure of stem rust pathogens

From 20 field samples from the two provinces of the country, 11 races were identified. The two adjacent regions, Khuzestan and Lorestan, had eleven similar races out of six and five races detected, respectively (Table 2). The highly virulent races of Ug99 (TTKSK) were the most abundant and widely distributed race across the Khuzestan region, with a frequency of 36.4%. The other abundant races countrywide included KTTSK and TTJQC with frequencies of 18.2% each. TRFSC and RRHSC made up the least dominant races in this region, with frequencies of 9.1% each. There was variation between the virulence spectra of races within the regions (Table 2). Of the 9 isolates studied in Lorestan, the highly virulent races TTJQC was the most abundant and widely distributed race across the region, with a frequency of 33.3%. Race TTKSK (Ug99) and the closely related race RRHSC were predominant, each with frequencies of

Table 1. Code for the 20 differential hosts for *Puccinia graminis* f.sp. *tritici (Pgt*) in ordered sets of five.

	Subset	Infection type produced on host lines with stem rust			
	1	Sr5	Sr21	Sr9e	Sr7b
	2	Sr11	Sr6	Sr8a	Sr9g
Pgt-Code	3	Sr36	Sr9b	Sr30	Sr17+13
	4	Sr9a	Sr9d	Sr10	SrTmp
	5	Sr24	Sr31	Sr38	SrMcn
B		Low*	Low	Low	Low
C		Low	Low	Low	High**
D		Low	Low	High	Low
F		Low	Low	High	High
G		Low	High	Low	Low
H		Low	High	Low	High
J		Low	High	High	Low
K		Low	High	High	High
L		High	Low	Low	Low
M		High	Low	Low	High
N		High	Low	High	Low
P		High	Low	High	High
Q		High	High	Low	Low
R		High	High	Low	High
S		High	High	High	Low
T		High	High	High	High

Source: Roelfs and Martens (1988); Jin et al. (2008); *Low: Infection types 0, 1 and 2 and combinations of these values. **High: Infection types 3 and 4 and a combination of these values.

Table 2. Races of *Puccinia graminis* f. sp. *tritici* identified in in Lorstan Province of Iran in 2012.

Resistance gene	Stem rust isolates collected in Lorstan Province								
	A1	A2	A3	A4	A5	A6	A7	A8	A9
5	3+	2+	3+	3+	2	2+	2+	3+	3+
21	3+	2+	3	2+	3	3+	2+	2	3
9e	3+	3+	3+	3+	3+	2-	2-	2	2
7b	33	33	33	3+	3+	2+	3+	3+	3+
11	3	2-	2;	2	3	2	1	2	2-
6	33+	33+	33	3+	3+	3+	33+	33+	3+
8a	3+	2+	3+	2+	2+	2+	2+	2	3+
9g	2	33+	3+	3+	3+	3+	3+	3+	3+
36	;C	1-	2-	1	2-	2-	;	2+	;C
9b	3+	2+	3+	2+	3+	3+	2+	2+	3+
30	33+	3+	2	33+	3+	33+	3	3	33
17	3	3+	3	3	3	3	3	3+	3+
9a	3+	3+	3+	3+	3+	3+	3+	33+	33+
9d	3+	3+	3+	3+	3+	3+	3+	3+	3+
10	33+	33+	3+	3+	3+	3+	33+	3+	3+
Tmp	2+	22+	3+	2+	2+	3+	2+	3+	2
24	2	1	2	;	2+	2+	2+	2	2
31	2	3	3+	3	3	3	3+	3	2
38	3	1-	1	2	3	2-	1	2-	3
McN	3+	3+	3	2+	3	3+	33	2	3+
Races	TTKSK	TTJQC	TRFSC	RRHSC	KTTSK	TTJQC	TTJQC	RRHSC	TTKSK

Table 3. Races of *Puccinia graminis* f. sp. *tritici* identified in in Lorstan Province of Iran in 2012.

Resistance gene	Stem rust isolates collected in Khuzestan Province										
	B1	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**	**B10**	**B11**
5	3+	3+	2+	3	2+	2	2+	2	3+	3+	+2
21	3+	3+	3	22+	3	3+	2+	2-	3	3+	2+
9e	3+	3+	3+	3+	33	2-	33	2+	2	33	3
7b	33	33	3+	3+	3+	2+	3+	3+	3+	3+	3
11	2+	2;	2;	2	3-	3	1	2	2-	3	1
6	33+	33+	33	3+	3+	3+	33+	33+	3+	3+	3+
8a	3+	3+	2+	3+	3+	2	2+	2+	2+	2	3+
9g	2	33+	3+	3+	3+	3+	3+	3+	3+	33+	3+
36	;	2-	2+	;1	2-	2-	;	2+	+2	1-	2+
9b	3+	3+	2+	3+	2+	3+	2+	2+	2+	2	3+
30	33+	3+	2+	33+	3+	33+	3	3	33	33+	3
17	3	3+	2	3	3	3	3	3+	3+	3+	3+
9a	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
9d	3+	3+	3+	3+	3+	3+	3+	3+	3+	3	3
10	33+	33+	3+	3+	3+	3+	33+	3+	3+	3+	3+
Tmp	2+	22+	22+	2+	3+	2+	3+	3+	2	3	22+
24	2	1	1	2	2+	2+	2	2-	2	22+	2-
31	2+	3	3+	3	3	3	3+	3	3	3	3
38	3	1-	1	2	1	2-	1	2-	3	2	2
McN	3+	3+	2-	3+	3	3+	2+	2	3+	3+	3
Races	TTKSK	TTKSK	TTJQC	TRFSC	RRHSC	KRKSC	TTJQC	TTJQC	KTTSK	TTKSK	KRKSC

22.2%, followed by races TRFSC and KTTSK with 11.2% each. From the Khuzestan region, races TTKSK (4 isolates), TTJQC (2 isolates), KTTSK (2 isolates), TRFSC (1 isolate), RRHSC (1 isolate) and KRKSC (1 isolate) were found.

Frequency in Lorestan province showed a similar trend, as TTJQC and TTKSK had the highest frequency of 33.3%. In this region, the least dominant races were TRFSC and RRHSC detected only once. The race pattern in Lorestan was different from that of the other region (Table 3). In two parts of the country, TTJQC and TTKSK were the dominant races, accounting for 33.3 and 36.4% of the total populations. Although most of the races were confined to specific locations, some had wider spatial distributions. Five races TTKSK (Ug99), TTJQC, TRFSC, RRHSC and KTTSK were present in two regions surveyed. KRKSC was present in southeast, but not in western Iran. Similarly, KRKSC was present in Khuzestan region (Table 2). In view of the results obtained (11 races from 20 samples), it is clear that race variations in stem rusts are very wide in Iran, and that with a sample size of only 20 isolates, only a minimal part of the races were actually found. In our study, more samples were collected from a lower area. This could be why lower races were identified in our study.

Virulence to Sr resistance genes

The majority of pathotypes identified during the survey were virulent on most of the wheat differentials. After analyzing 20 isolates from regions representing the major wheat-growing areas of the country, two important stem rust resistance genes, namely *Sr24* and *Sr36* were found to confer resistance to most of the races prevalent in two provinces of Iran. Three resistance genes, *Sr11*, *Sr38* and *SrTmp*, were found as low infection types (more effective resistance genes) against most of the races detected in this study (Table 4). Race TTKSK, was virulent to all except three resistance genes *Sr36, Sr24* and *Sr38*. Among these 11 races, race KTTSK thus poses a serious threat to two provinces of the country. Race KRKSC, which had a wide spectrum of distribution (Table 4) was virulent to all differentials except those carrying the Sr24 and Sr36 genes. The resistance genes *Sr5, Sr8a, Sr9e, Sr9b* and *Sr21* were found to be effective against races TTJQC and RRHSC while genes *Sr6, Sr7b, Sr9g, Sr17, Sr30, Sr9a, Sr9d, Sr10,* and *SrMcn* were ineffective for all races identified in Khuzestan region. A total of 5 different races were identified from 9 stem rust isolates in the Lorestan province.

Among these 5 races, Race TTKSK, was virulent to all except four resistance genes *Sr24, Sr36, Sr38* and *SrTmp* while resistance genes *Sr24* and *Sr36* were effective against race KTTSK (Tables 2 and 3). In general, the resistance genes *Sr11*, *S24*, *Sr36, Sr38* and *SrTmp* showed resistance or low infection types against all isolates, while genes *Sr5, Sr6, Sr9a, Sr10* and *SrMcn*

Table 4. Virulence spectrum and frequency of races of *P. graminis* f. sp. *tritici* collected from two provinces of Iran.

Race	Ineffective resistance genes[b]	Location	No. of isolates	Frequency (%)
Khuzestan				
TTKSK	*5*, *6, 7b, 8a, 9a, 9b, 9d, 9e, 10*, *11*, *17, 21, 31,38, Tmp*, *McN*	Ahvaz	4	36.4
TTJQC	*5* , *6, 7b, 9a, 9b, 9d, 9e, 9g, 10, 17, 31, Tm,*	Dezful	2	18.2
TRFSC	5 **,** *6, 7b, 8a, 9a, 9b, 9d, 9e, 9g, 10, 30, 31*, *McN*	Behbahan	1	9.1
RRHSC	*6*, *7a, 7b,* 8a, *8b, 9g, 9a,* 9b, *9d, 9e, 10, 11, 13, 17,* 30, 31, *Tmp, McN*	Shushtar	1	9.1
KRKSC	*6, 21, 7b, 7a, 8b, 9e, 9g, 9a, 9d, 11, 13, 17, 21, McN 10,*	Shadegan	1	9.1
KTTSK	5 *, 6, 9a, 9b, 9d, , 9g, 10, 17, 31*, *38*, *McN*	Izeh	2	18.2
Total			11	100
Lorestan				
TTKSK	5*, 6, 7b, 8a, 9a, 9b, 9d, 9e, 9g, 10, 11, 17, 21, 31,38, McN*	Khorramabad	2	22.2
TTJQC	*6, 7b, 8a, 9a, 9b, 9d, 9e, 9g, 10, 17, 21, 31, Tmp*, *McN*	Aligoodarz	3	33.3
TRFSC	*5, 6, 7b, 8a, 9a, 9b, 9d, 9e, 9g, 10, 17, 21, 30*, *31, Tmp*, *McN*	Dorood	1	11.2
RRHSC	5*, 6, 7b, 8a, 9a, 9b, 9d, 9e, 9g, 10, 17, 21, 30*, *31, Tmp, McN*	Borujerd	1	22.2
KTTSK	*6, 7b, 8a, 9a, 9b, 9d, 9e, 9g, 10,11, 17, 21, 30, 31, 38, Tmp, McN,*	Koohdasht	2	11.2
Total			9	100

showed high infection types against all isolates in 2012 (Table 4). Only two of the 20 differential lines carrying resistance genes, *Sr24* and *Sr36* were found to confer resistance to most of the races prevalent in two provinces of the country.

DISCUSSION

The identification of 11 races from 20 samples was a clear indication of high virulence diversity within the *Pgt* population in two provinces of country. A comparison of the races identified in the present study with earlier reports (Nazari et al., 2008 and 2009) revealed some differences.

Even though stem rust incidences were relatively high in both provinces, the percentage of plants infected with stem rust in diseased fields was low. The number of fields inspected during our study was different (Tables 2 and 3). The highest stem rust pathogens was found in a field of Khuzestan province. Although Khuzestan and Lorstan provinces have climates that favor rust development, the diseases were not apparent.

It is also important to note that the race spectrum in Iran is clearly different from that reported in other parts of the world. Surveys in the USA (Jin et al., 2007, 2008) detected fewer races, than in Iran. According to our reports, out of the 20 field samples from the two provinces of the country, 11 races were identified. Race TTKSK, which had a wide spectrum of distribution was virulent to all differentials except those carrying the *Sr24, Sr36* and *SrTmp* genes.

Since it is not known which resistance genes local landraces and commercial wheat cultivars grown in Iran contain, it is not possible to determine their effect on race composition of the *Pgt* population. The two adjacent regions, Khuzestan and Lorstan, had six similar races out of five races detected, respectively. The present study also detected the race at one additional location, indicating that the race is spreading in the region. Furthermore, the new Ug99 variant TTKSK, which is identified in this study, was also detected in other countries. Positive Iranian Ug99 isolates were collected in 2007 from two sites, Borujerd and Hamadan, in northwestern Iran, but underwent extensive testing to confirm the race (Nazari et al., 2008). Detection in Iran in 2007 was followed by drought conditions, and no reports on Ug99 were received from Iran in 2008 (Nazari et al., 2009). However, in 2012 Ug99 was found in the southern Iranian province of Khuzestan, where spring wheat is grown and growing conditions are favorable. Alternatively, Ug99 may have been introduced into Khuzestan in 2007 but remained undetected and migrated to the northwest, where facultative and winter wheats are grown and mature approximately two months later. There is no evidence that the Ug99 lineage has become well established in Iran, and no crop losses have been reported so far. Also, to date it is not known to have spread beyond Iran.

Reports of the unusual occurrence of stem rust in Pakistan in 2009 prompted some fears of a Ug99 incursion, but greenhouse phenotyping of samples on differentials and DNA analysis of dead spores indicated conclusively the absence of Ug99 and presence of another important race (RRTTF) (Mirza et al., 2010).

In general, the virulence spectrum of the pathogen in this study confirmed the presence of wider range of virulence in the study area and is in line with previous studies conducted in Iran (Patpour et al., 2011; Nazari et al., 2008). A comparison of the races identified in the present study with these earlier reports revealed differences. This could be due to variation in location and time, as the prevalence of races in a specific season and region depends on the type of wheat cultivars grown and to some extent on the predominant environmental conditions, especially temperature (Roelfs et al., 1992). It was evident that the majority of the resistance genes were ineffective against most of the isolates. Resistance genes *Sr5, Sr6, Sr7b, Sr9g, Sr17, Sr30, Sr9a, Sr9d, Sr10* and *SrMcn* were ineffective, accounting for more than 65% of the isolates tested. Nazari et al. (2009) reported similar findings. These *Sr* genes were ineffective for more than 85% of the isolates collected in 2007 from northwest regions of Iran. Earlier studies indicated that virulence to *Sr6*, *Sr8b*, *Sr9a*, *Sr9d* and *Sr11* is common worldwide (Roelfs et al., 1992). In contrast, *Sr24* was effective against most of the isolates tested. This confirms the report of Patpour et al. (2011), which stated that these genes are amongst the effective genes, which have an adequate and some immediate values against almost all races in the world, except few occasional high infection types in some countries including Iran. For instance, virulence to *Sr24* was reported in Kenya in 2006. A variant of Ug99 that added virulence on stem rust gene *Sr24* (Ug99 + *Sr24* virulence, called TTKST) has further increased the vulnerability of wheat to stem rust worldwide (Jin et al., 2008). On the other hand, Admassu et al. (2009) reported that race TTKSR was avirulent on *Sr24*. Teklay et al. (2012) reported that *Sr24* was effective against many races, however, in our study, this genotype was effective fir most isolates tested.

Nazari et al. (2009) previously detected a *Pgt* isolate virulent to *Sr31* in BorooJerd, but the lack of stem rust in Iran during 1997-2006 due to unfavorable climatical conditions hindered more investigation on virulence factors of *Pgt,* therefore statement on virulence for *Sr31* in the region during last ten years is rather difficult. However, virulence for *Sr31* should be considered as serious threat to wheat production in Iran.

The results of this study also support this fact and show that large variation was observed in terms of the determined races. In general, it appears that many resistance genes have different reactions to the races, and our results confirmed that there are many stem rust races in Iran. The geno-types possessing at least one of the resistance genes *Sr11*, *Sr24*, Sr36, *Sr38* and *SrTmp*, were resistant or found with low infection types against most of the races detected in this study (Tables 2, 3 and 4). Each of these resistance genes or a combination of them could be used in developing stem rust resistant cultivars. On the other hand, the absence of virulence towards *Sr6, Sr7b, Sr9g, Sr17, Sr9a, Sr9d, Sr10, Sr30* and *Sr31* genes in all rust races studied during the two provinces of country indicate that these genotypes could serve as a source of resistance to the prevailing rust races in Iran.

Conflict of Interest

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

ACKNOWLEDGEMENTS

We thank Dr. Kurt J. Leonard from Department of Plant Pathology, University of Minnesota St. Paul, USA for his discussions and suggestions, and for providing certain materials for the study. Financial support and the provision of facilities from the Islamic Azad University, Shoushtar Branch for this research is gratefully acknowledged.

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Vol. 8(47), pp. 3837-3849, 19 November, 2014
DOI: 10.5897/AJMR2014.7090
Article Number: FD0E75948998
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Optimization of parameters for decolorization of a textile azo dye, Remazol Black B (RBB) by a newly isolated bacterium, *Bacillus thuringiensis* BYJ1

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Received 28 August, 2014; Accepted 12 November, 2014

***Bacillus thuringiensis* BYJ1, a newly isolated bacterium from textile effluent contaminated soil was exploited to decolorize a textile azo dye, Remazol Black B (RBB). Various process parameters like initial RBB concentration, temperature, pH and cultural conditions on the process of dye decolorization were studied in order to determine optimum condition. Decolorization process was largely affected under aerobic process. Despite the toxic and inhibitory effects of dye, *B. thuringiensis* BYJ1 was able to tolerate as high as 1200 mg L^{-1} of RBB. Optimum decolorization of RBB was achieved at 37°C and pH 7 under static culture condition. Decolorization process was also studied using immobilized cells of *B. thuringiensis* BYJ1. The decolorized dye products were analyzed by thin layer chromatography (TLC), UV-visible scanning and fourier transform infrared spectroscopy (FTIR) analysis. Seed germination assay was performed to analyze the effect of decolorized dye products. The plasmid isolation and its curing from *B. thuringiensis* BYJ1 was performed to study effect of plasmid on the process of biodegradation of RBB.**

Key words: Azo dye, decolorization, immobilization, plasmid curing, *Bacillus thuringiensis*.

INTRODUCTION

Application of metabolic potentials of microbes in remediation of polluted sites is an efficient, effective, economic and eco-friendly way to solve the pollution related environmental problems. In bioremediation, we utilized microorganisms and/or their enzymes as tools to degrade both natural and anthropogenic materials in wastewater digesters, composters, landfills, natural terrestrial environments and natural or artificial aquatic ecosystem as an advanced bioremediation technology.

Synthetic dyes are vital for the textile dyeing and printing industries. Amongst all synthetic dyes, azo dyes are the most common, being used up to 90%. There are more than 8000 chemical products associated with the dyeing process listed in the Colour Index (Society for Dyes and Colourist, 1976) while more than 1,000,000 commercially available dyes exist with more than 7 x 10^5

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metric tons of dyestuff produced annually (Meyer, 1981; Zollinger, 1987). During dying process, almost 10-15% of the dyes are lost as component of textile effluent (Vaidya and Datye, 1982) and due to this, the pollution by dye-waste water has reached an alarming level. There are several dyes that exhibit toxic and mutagenic effect on aquatic as well as other living systems (Brown and Stephen, 1993). Sewage treatment plants that can deal with different kinds of sewage have been developed; however, technology is still unable to treat many chemicals. More worrying is the fact that current legislation only governs the amount of acidity, alkalinity, COD, amount of biochemical oxides of industrial effluents, but not the dye concentrations and extent of degradation of the compounds.

Azo dyes are the most important commercial colorants because of their wide color range, good fastness properties and pictorial strength as compared to anthraquinones dyes, the second most important group of the dyes. These include several structural varieties of dyes, such as acidic, reactive, basic, disperse, azo, di-azo, anthrax-quinone-based and metal-complex, etc. The only thing in common is their ability to absorb light in the visible region of electromagnetic radiations.

The dye can be primarily removed from the textile effluent by adsorption (biosorption), precipitation and by microbial biotransformation and/or biodegradation in different conditions (Brown and Laboureur, 1983; El-Gundi, 1991). There are reports on bioremediation of textile azo dyes (reactive and disperse dyes) Direct Blue, Procion Navy Blue, Procion Green and Supranol Red by a bacterial consortium consisting of known strains of *Pseudomonas* and *Bacillus* (Asgher et al., 2007; Abraham et al., 2004). The mechanism and kinetics of process of azo dye biodegradation by a microbial consortium consisting of bacteria and white rot fungus was studied by Fang et al. (2004). This may help to manipulate the pathway of biode-gradation or increase the rate of reaction by manipulating the kinetics parameters. There are various reports of microbial degradation of textile and laboratory dyes by fungi, actinomycetes, yeast, algae and bacteria (Patricia et al., 2005; Xu et al., 2007). Still the urge of researchers to find the new strains of bacteria with dye decolorizing capacities has not come to an end. The dye decolorizing capacity of indigenous bacteria, like *Bacillus* sp.*, Klebsiella, Planococcus* sp., *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, were optimized to generate effective process to degrade different dyes (Joe et al., 2011; Mohan et al., 2013; Bhatnagar et al., 2013; Shah et al., 2013). Recently, the area of focus in the research is to find or design the group of bacteria involved collec-tively in the textile dyes degradation as a co-metabolism approach of a natural process and consortium studies at the laboratory levels (Mahmood et al., 2012; Neelambari et al., 2013).

The development of a large scale establishment ofbiore-mediation technology which should be efficient, economic and fast is the current demand of the developing countries like India.

The present study was focused on isolation, screening and identification of textile dye decolorizing bacteria from contaminated sites. Attempts were made to check the decolorization efficiency under free cells and immobilized cell condition. Process parameters were tested in order to optimize the condition of dye decolorization by isolated bacteria.

MATERIALS AND METHODS

Sample collection

Soil and effluent samples were collected from the dying and printing industry effluent release sites of Vatava, Ahmedabad, Gujarat, India. The Vatava GIDC is one of the largest industrial area of Gujarat state hosting more than 1200 dye manufacturing units. Autoclaved sampling bags were used to collect soil samples stored at 4°C till used.

Dyes, culture media and chemicals

The Remazol Black B (RBB - λmax 595nm) was procured from local dye manufacturing industries situated at Vatava, Ahmedabad, Gujarat (India). Other chemicals and medium components used in this study were of analytical and molecular grades.

Nutrient Broth (N-broth) purchased from Hi-Media, Mumbai consisted of (g L^{-1}): Peptone (5), yeast extract (1.5), beef extract (1.5), NaCl (5) with pH 7.4 ± 0.2. Nutrient Agar (N-agar) purchased from Hi-Media, Mumbai consisted of (g L^{-1}): Peptone (5), yeast extract (1.5), beef extract (1.5), NaCl (5), Agar (15) with pH 7.4 ± 0.2. Minimal salt medium (MSM) consisted of (g L^{-1}): KH_2PO_4 (2), K_2HPO_4 (0.8), $MgSO_4.7H_2O$ (0.1), NaCl (0.1), $FeSO_4.7H_2O$ (0.02), NH_4NO_3 (1.0), $CaCl._2H_2O$ (0.02) and glucose (0.5). The pH of the medium was adjusted to 7.0 ± 0.2.

A stock solution of RBB (1% w/v) was prepared and autoclaved at 121°C and 15 lbs for 10 min and stored at 4 - 8°C temperature. Required dilutions were prepared from this stock for further use.

Enrichment and isolation of dye decolorizing bacteria

Isolation of dye decolorizing bacterial was carried out by inoculating 1 g of contaminated soil in 500 ml of Erlen Meyer flask consisting 200 ml of MSM supplemented with RBB (500 mg L^{-1}) as a sole carbon source. The flasks were incubated on orbital shaker at 120 rpm for 30 days. After every 5 days of incubation, a loop-full of enriched medium was streaked on N-agar plates and incubated at 37°C for 24 to 72 h. Simultaneously, 1 ml of enriched culture was transferred to fresh MSM medium supplemented with RBB as described earlier. Such serial transfer was performed for 30 days. Isolation of bacterial strain was performed by successive streaking on nutrient agar plate supplemented with RBB till single isolated pure colony was obtained.

Screening of dye decolorizing bacteria

The primary screening for dye decolorization ability of all bacterial isolates was carried out using solid MSM-agar containing RBB

(500 mg L^{-1}). The actively growing cultures of individual isolates were spotted in the center of the agar plates and the plates were incubated at 37°C for 48 h. The organisms which showed bigger zone of decolorization were subjected to secondary screening process.

The secondary screening process was performed in liquid medium. Overnight grown culture of 0.5 OD (1%) was used to inoculate 100 ml N- broth supplemented with Remazol Black B dye (100 mg L^{-}). The inoculated flasks were incubated under static conditions for 96 h at 37°C. Samples were withdrawn every 12 h from the flasks and decolorization assay was performed to determine the decolorization activity.

Identification and phylogenetic analysis of *B. thuringiensis* BYJ1

The isolated dye decolourizing bacterium BYJ1 was identified by 16S rRNA gene sequence analysis. Genomic DNA was isolated from the pure culture pellet. The ~1.5 kb 16S rRNA gene fragment was amplified by high-fidelity PCR polymerase using consensus primers. The PCR products were cloned in plasmid and plasmid DNA was sequenced bi-directionally using the forward, reverse and an internal primer specific for bacterial domain. Sequence data was aligned and analyzed for finding the closest homologous for the query sequence. The nucleotide sequence analysis of the sequence was performed by BLASTN (Basic Local Alignment sequence Tool) site at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). The alignment of the sequences was performed by using CLUSTALW program at European Bioinformatics site (www.ebi.ac.uk/Tools/clustalwomega) using the neighbor joining algorithm. A phylogenetic tree was constructed from the alignment by average distance of the percentage identity, using Jalview. A second clustering was done with the MEGA 5.2 software, with the same algorithm. After 500 iterations of bootstrapping, the BYJ1 strain was clustered in the *Bacillus* group with a bootstrap value of 98 (98% repeatability of the clustering). The sequence was refined manually after crosschecking with the raw data to remove ambiguities and was submitted to the NCBI.

Decolorization assay

The samples collected from all reaction mixtures were centrifuged at 8000 xg, 4°C, 20 min (REMI, RM-1214) and supernatants were used to determine the percentage decolorization. The decolorizing activity was determined by monitoring the decrease in absorbance at 595 nm (λ_{max} of RBB). The un-inoculated flasks containing dye was used as control. Decolorization activity (%) was calculated as: Decolorization activity (%) = [(A – B)/ A] x 100. Where, A = initial absorbance and B = observed absorbance.

Effects of process parameters on decolorization of RBB by *B. thuringiensis* BYJ1

Effects of pH and temperature on decolorization of RBB

Nutrient broths containing 100 mg L^{-1} RBB were prepared in 250 ml Erlenmeyer flask using different buffer solutions. The buffers selected for media preparation were 0.1 M citrate buffer (pH 4 and 5), 0.1 M sodium phosphate buffer (pH 6 and 7) and 0.1 M Tris buffer (pH 8, 9 and 10). Fresh nutrient broths were inoculated with actively growing culture of *B. thuringiensis* BYJ1 (1% v/v) and incubated under static culture conditions.

Nutrient broth with pH 7 was used for testing effect of temperature on the process of dye decolorization. Young culture of *B. thuringiensis* BYJ1 was inoculated in 100 ml of nutrient broth containing 100 mg L^{-1} RBB and incubated at 10, 20, 30, 37, 40, 50 and 60°C temperature under static culture conditions. The decolorization activity for both sets was monitored at 0, 24, 48 and 72 h of incubation.

Effects of dye concentrations

Various concentrations of RBB (50, 100, 200, 400, 600, 800, 1000 and 1200 mg L^{-1}) were prepared in different reaction mixture flasks and inoculated with actively growing culture of *B. thuringiensis* BYJ1 (1% v/v). The flasks were incubated at 37°C and incubated under static culture condition. Decolorization activity was measured at 0, 24, 48 and 72 h of incubation time as described above.

Biodegradation of RBB and analysis of the transformed dye products

Nutrient broths containing RBB (100 mg L^{-1}) inoculated with actively growing culture of *B. thuringiensis* BYJ1 (1% v/v) were incubated at 37°C under static and shaking culture conditions (120 rpm) for 96 h. The decolorized dye samples were harvested after every 12 h and percentage decolorization was calculated. Samples harvested after 96 h were subjected to different analysis.

UV–visible spectroscopic analysis

The supernatants were scanned in Picodrop® UV-Visible spectrophotometer at Department of Biochemistry, Anand Agriculture University, Anand, Gujarat, India.

FTIR and TLC analysis of the transformed dye products

The cell free supernatants obtained after decolorization under static and agitated culture conditions were extracted with equal volume of ether and dried. The concentrated samples were dissolved in methanol and used for FTIR analysis. The FTIR analysis of extract was performed using standard potassium bromide solution.

The samples were analyzed on TLC plates (MerK. GaA - 64271, Darmstadt, Germany). The solvent system used consisted of n-butanol : acetic acid : water (4:2:4 v/v). The resolved chromatogram was observed under natural light, short wave length UV (254 nm) and long UV (365 nm). The R_f values of the dye spot and newly immersed spots were calculated using following formula:

R_f = Distance traveled by solute/distance traveled by solvent

Seed germination study

The effect of dye and its degradation products on seed germination was analyzed by irrigating seeds with decolorized dye under static and agitated culture conditions. Seeds of mung (*Phaseolus mungo*) and groundnut (*Arachis hypogaea*) were surface sterilized by 0.1 % w/v $HgCl_2$ solution and rinsed in sterile distilled water to remove residual $HgCl_2$. The seeds were incubated in sterile tubes and were irrigated with different treated dye supernatants of static and shaking samples along with the water and Remazol Black B dye (100 mg L^{-1}) solution as controls for 13 days.

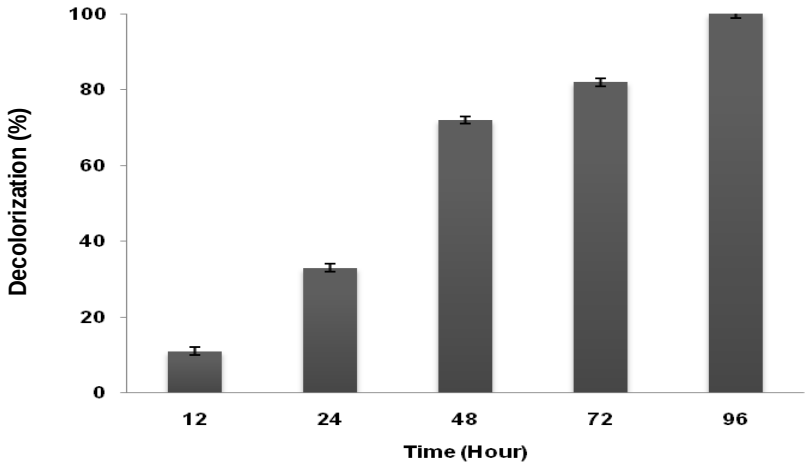

Figure 1. Decolorization of Remazol Black B by *B. thuringiensis* BYJ1.

COD removal and decolorization of RBB

Chemical oxygen demand (COD) analysis was carried out as per Standard Methods for the Examination of Water and Wastewater, APHA (Andrew et al., 1999). 250 ml Erlenmeyer flask containing 100 ml N-broth with RBB (100 mg L^{-1}) was inoculated with 1% v/v inoculum of *B. thuringiensis* BYJ1 and incubated at 37°C under static culture condition for 96 h. Inoculated (media + BYJ1) and uninoculated (media + RBB) controls were also included. Samples were harvested at intervals and analyzed for decolorization.

Interaction of RBB and B. thuringiensis BYJ1

Erlenmeyer flask (250 ml) containing 100 ml Nutrient broth supplemented with RBB (100 mg L^{-1}) was inoculated with 1% v/v actively growing culture of *B. thuringiensis* BYJ1 and incubated at 37°C. After 24 h incubation under agitated culture condition, the broth was analyzed by scanning electron microscopy to examine the interaction of RBB with *B. thuringiensis* BYJ1.

Decolorization of RBB by immobilized cells of *B. thuringiensis* BYJ1

B. thuringiensis BYJ1 was encapsulated in alginate beads and its efficiency for RBB decolorization was measured. The alginate beads were prepared using sterile solutions of sodium alginate (3% w/v), calcium chloride solution (5% w/v) and young culture of *B. thuringiensis* BYJ1 (1.6 x 10^7 CFU/ml). Nutrient broth containing RBB (100 mg L^{-1}) was inoculated with beads of immobilized *B. thuringiensis* BYJ1.

Role of *B. thuringiensis* BYJ1 plasmid in decolorization of RBB

The bacterial isolates were grown in the presence and absence of dye Remazol Black B (100 mg L^{-1}) in N-Broth for 24 h at 37°C. The cell pellets were obtained from the samples collected from both the reaction mixture.

The plasmids extraction was performed by modified alkali lysis methods from the culture grown in the presence and absence of dye. The isolated plasmid samples were subjected to agarose gel electrophoresis. The process was performed using 1% w/v agarose, TAE buffer and ethidium bromide (EtBr) to stain the gel for the visualization of plasmid DNA (Sambrook and Russell, 2001).

Plasmid curing of B. thuringiensis BYJ1 using acridine orange

The activated culture of *B. thuringiensis* BYJ1 was used for plasmid curing. The 100 ml sterile N-broth was prepared in which acridine orange was added at a final concentration of 100 µg/ml. The media was inoculated with activated culture of *B. thuringiensis* BYJ1 (1 % v/v) and was incubated at 37°C for 48 h. After 48 h incubation, 100 µl of sample was withdrawn and serial dilution was carried out. The diluted samples were spread N-agar plates and plates were incubated at 37°C for 24 h. After 24 h, a replica of all the plates with isolated colonies were prepared using N-agar with Ampicillin (10 µg) plates and replica plates were incubated at 37°C for 24 h. After incubation, the antibiotic plates were compared with master plates and colonies which were absent on antibiotic plates were selected from master plate.

The plasmid cured colonies were subjected to plasmid isolation and agarose gel electrophoresis for conformation.

RESULTS AND DISCUSSION

Screening and isolation

seventy nine different types of bacterial strains were isolated through enrichment techniques. Among these 79 isolates, few showed less and some showed moderate decolorization activity during the screening procedures. A few of them decolorized dyes completely. The bacterial isolate C49 was one of them and the complete decolorization of Remazol Black B was achieved in 96 h (Figure 1).

Molecular identification of BYJ1

Based on 16S rRNA gene nucleotides homology and phylogenetic analysis, the bacterium, BYJ1 (Gene Bank

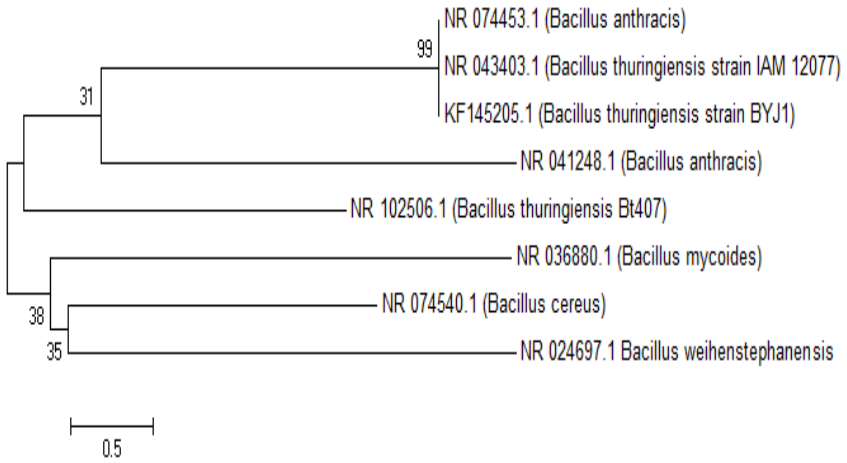

Figure 2. Phylogenetic tree of *B. thuringiensis* strain *BYJ1*.

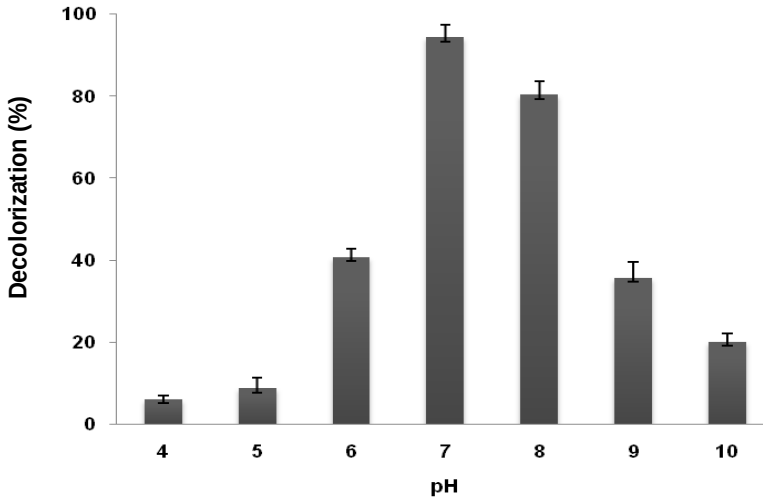

Figure 3. Effect of pH on the decolorization process of RBB by *B. thuringiensis* (BYJ1).

Accession Number: KF145205; *Bacillus thuringiensis* BYJ1) was identified as *B. thuringiensis*.

Genomic DNA was isolated from the pure culture and using consensus primers, the ~1.5 kb 16S rDNA fragment was amplified using *Taq*DNA Polymerase. The PCR product was bi-directionally sequenced using the forward, reverse and an internal primer; sequence data was aligned and analyzed for finding the closest homologs for the microbe. The phylogenetic tree was drawn to scale, with the branch length in the same unit as those in evolutionary distance (Dhanve et al., 2009). The phylogenetic tree of *B. thuringiensis* BYJ1 (Figure 2) shows that the nearest homologous species was found to be *Bacillus anthracis* (Accession No. NR_074453).

Effects of pH and temperature on decolorization of RBB

The pH is one of the important factors affecting the metabolic activities of the organisms. As shown in Figure 3, the dye decolorization activity of *B. thuringiensis* BYJ1 was optimum at pH 7.0. However, *B. thuringiensis* BYJ1 could resist and retain its ability to decolorize Remazol Black B at pH 8.0 also. This is promising feature because in India, most of the dye or textile effluents are neutralized by lime treatment before they enter to the biological treatment.

The temperature is another critical and important factor that affects growth and metabolism of an organism. As shown in Figure 4, after 96 h incubation at different

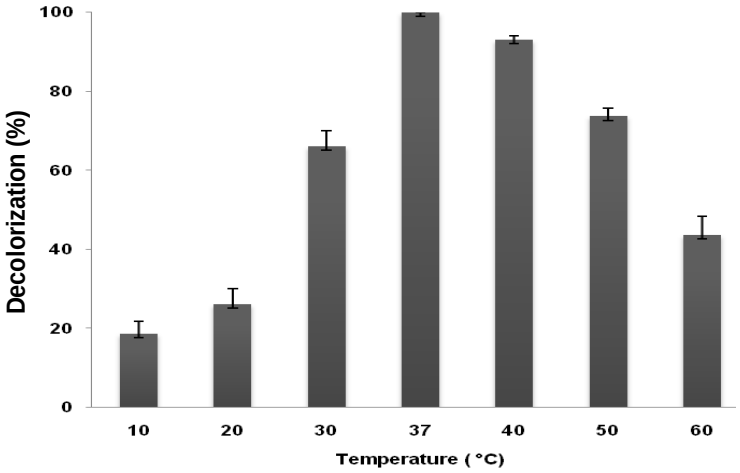

Figure 4. Effect of temperature on the decolorization process of RBB by *B. thuringiensis* BYJ1.

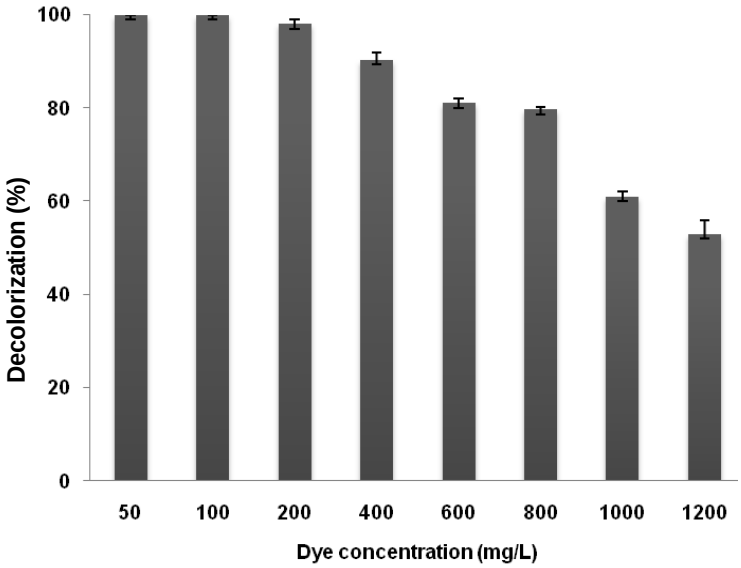

Figure 5. Effect of initial dye concentration on the decolorization process of RBB by *B. thuringiensis* BYJ1.

temperature, the dye decolorization activity of *B. thuringiensis* BYJ1 was retained till 60°C. Notably the increase in temperature above 37°C decreased the dye decolorization efficiency of *B. thuringiensis* BYJ1. This can be attributed to loss of cell viability or inactivation of enzymes at relatively high temperature (Kapil et al., 2009).

Effects of RBB concentrations

Very few organisms, especially bacteria, can tolerate textile dyes in high concentrations. The high concentration of dye may affect the growth and metabolism of an organism. Many times the concentration of textile dye above certain range proved to be toxic to the organisms (Bhimani et al., 2014). The mode of toxicity has not been characterized till now. There are different believes among different groups of scientists.

As shown in Figure 5, *B. thuringiensis* BYJ1 could tolerate Remazol Black B dye up to 1200 mg L^{-1}, but the decolorization ability of *B. thuringiensis* BYJ1 was not affected much up to 200 mg L^{-1} dye concentration and was giving 100% decolorization of dye. The increase in

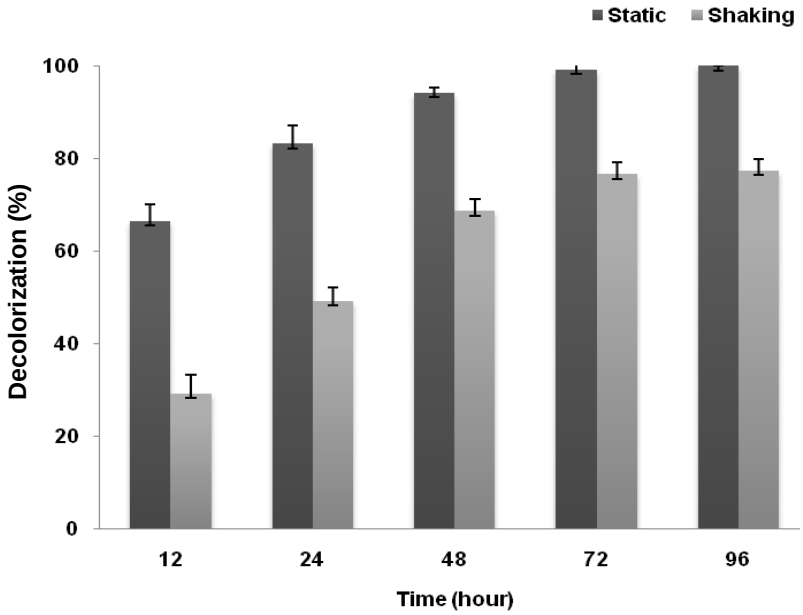

Figure 6. Effect of aeration/agitation on the decolorization process of RBB by *B. thuringiensis* BYJ1.

dye concentration decreased the decolorization efficiency of *B. thuringiensis* BYJ1 up to 50%.

Biodegradation of RBB and analysis of the transformed dye products

The reviews on azo dyes decolorization show that anaerobic biodegradation of azo dyes led to production of aromatic amines which are much more carcinogenic than the dye itself. That is why the aerobic biological treatment of dye degradation is preferred over anaerobic treatment but aerobic decolorization of dye is carried out by very few groups of organisms. The anaerobic treatment is powerful and faster as compared to aerobic treatment but its products are not acceptable to the receiving environment (Puvaneswari et al., 2006).

As shown in Figure 6, the complete decolorization of Remazol Black B was achieved in static condition after 96 h incubation while in shaking condition only 77% dye was decolorized.

UV-visible spectroscopic analysis

Remazol Black B dye control sample was analyzed for UV-visible scanning and showed peak at 595 nm. Samples were collected from static (ST) and shaking (SH) reaction mixture and centrifuged to remove biomass. Supernatant was subjected to UV-visible scanning and the peak was significantly reduced in both the samples as compared dye control. Static sample showed a new peak in UV range indicating existence of breakdown products of RBB.

FTIR analysis of the transformed dye products

The FTIR spectra obtained from the treated dye samples showed several peaks in the region where N–H and O–H stretching is normally observed, like 3318.68 and 3319.93 cm^{-1} respectively, in Figures 7 and 8. The sample from aerobic (shaking) flask showed significant reduction in absorption and so reduction in peak size at 3300 cm^{-1} region as compared to the sample from microaerophilic static condition corresponding to –NH- (stretching) in primary amine. In both spectrum, there is no peak found in 1590 – 1600 cm^{-1} region which is fingerprint region for N=N stretching for azo bond (Figures 7 and 8). The absence of peak in the above region indicates breakdown of azo bonds of diazo dye Remazol Black B (Lamia et al., 2009).

Other band located at 1658.82 cm^{-1} in the spectra of static sample (Figure 7) disappeared in aerobic treatment (Figure 8). The significant peak at 1658.82 cm^{-1} is for NH_3^+ deformation and suggest that possible alkenes conjugation with C=O and -C-N stretching vibration regions and it is well established that microaerophilic or anaerobic treatment of azo dyes produces aromatic amines. Moreover, no absorption peak in this region of the spectra of aerobic sample (shaking condition) indirectly indicates absence of such amines.

In both samples peaks at 595.35 and 625.22 cm^{-1} shows production of sulfo-compounds as the region specifies bending vibration of S=O bond. The peaks at

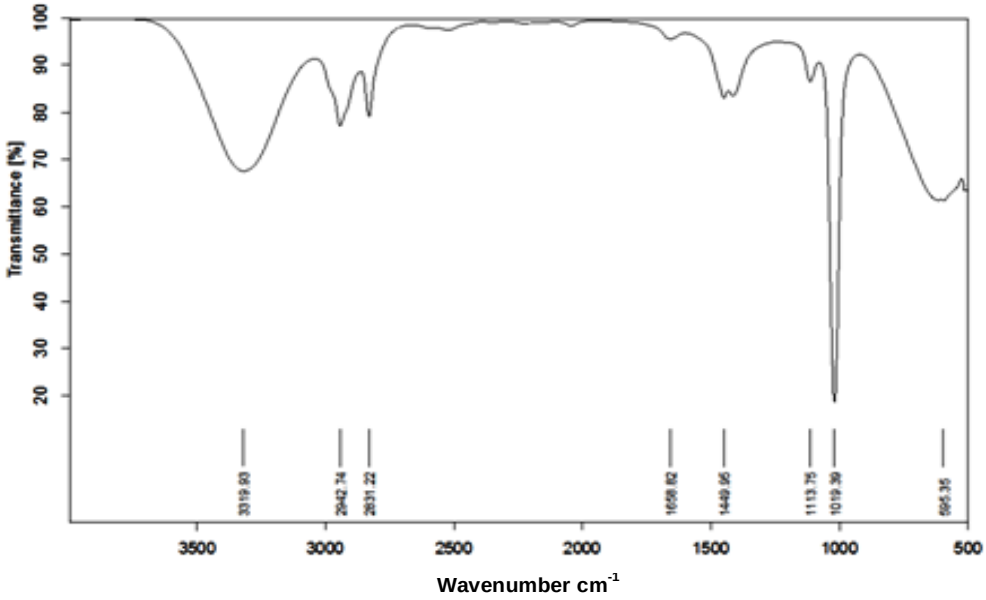

Figure 7. FTIR spectrum of decolorized RBB dye by *B. thuringiensis* BYJ1 under static condition.

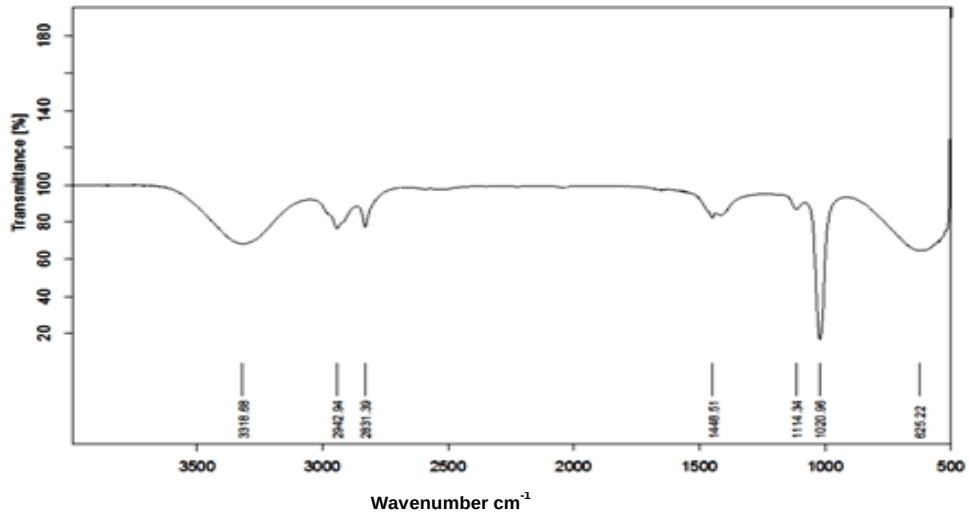

Figure 8. FTIR spectrum of decolorized RBB dye by *B. thuringiensis* BYJ1 under shaking condition.

1019.39 and 1113.75 cm^{-1} in static sample spectrum (Figure 7) and the peaks at 1020.96 and 1114.34 cm^{-1} in shaking sample spectrum (Figure 8) were for C-H deformation. The peaks at 1449.95 cm^{-1} in static spectrum and 1448.51 cm^{-1} in shaking spectrum were for alkanes C-H deformation. The peaks at 2831.22, 2831.39, 2942.71 and 2942.94 cm^{-1} were indicating alkanes C-H stretching.

There was considerable difference between the FTIR spectrum of treated samples in static and shaking condition in terms of reduction in peaks' size and disappearance of peak. This indicated biodegradation of dye through two different ways due to difference in cultural conditions.

The fact was better conformed by toxicity testing of these samples on seed germination studies. If the organism uses same pathway for the degradation of Remazol Black B under both conditions, then the samples must have the same effect on seeds germinations.

Table 1. Seeds germination studies of dye samples.

Sample	Seed germination (%)	
	Phaseolus mungo	***Arachis hypogaea***
Water control	96	90
Dye control	22	09
Shaking sample (degraded dye)	98	84
Static sample (degraded dye)	69	37

Thin layer chromatography (TLC)

The decolorization was further conformed by analyzing the static and shaking sample on TLC plates. When TLC plates were observed under natural light, short wave length UV (254 nm) and long UV (365 nm), no spot was found corresponding to the dye spot (dye control having R_f 0.66). This means that the textile dyes can be decolorized and degraded in both conditions.

Seed germination studies

Treated effluents of shaking and static conditions were subjected to seed germination studies using *Phaseolus mungo* and *Arachis hypogaea*. The sample treated in shaking condition showed almost equal seed germination capacity as with positive control water (Table 1). In contrast, the sample of the static condition was having less favorable effect on seeds germination. Dye solution which was used as negative control affecting negatively the germination of seeds (Patil et al., 2008). The biologically treated dye effluent under shaking condition can be used for irrigation purpose after suitable post treatment while statically treated effluents requires few more steps of treatment before disposal in the receiving body.

COD removal and decolorization of RBB

The treatments of environmental samples are done with the intention of reduction in COD and/or biochemical oxygen demand (BOD) values. The efficiency of any waste water treatment technology is finally assessed on the basis of its ability to reduce COD of the samples.

The initial COD load of the reaction flasks consisting of 100 mg L^{-1} Remazol Black B was 18051 ppm. The COD of samples was decreased when treated with *B. thuringiensis* BYJ1, after 24 h of incubation there was 4076 ppm reduction in COD. After 48 h, 7844 ppm reduction was found with 70% dye decolorization and finally total 16087 ppm reduction on COD was achieved with 99% total dye decolorization.

Interaction of dye Remazol Black B with *B. thuringiensis* BYJ1

The scanning electron micrograph of the organism in the presence and absence of dye revealed that in the presence of dye the thickness of the microbial cell increases significantly therefore dye Remazol Black B may get adherence on the surface of the *B. thuringiensis* BYJ1 and then must be degraded in small units.

It was found that when the isolates were grown in the presence of dye, their cell dimensions changed. From the electron micrographs, it was clear that during the decolorization process the dye gets deposited on the cell surface of the organisms. The decolorization may be done by membrane associated systems of decolorization and then simplified molecules of dyes may be internalized and further degraded. The cell length of *B. thuringiensis* BYJ1 was measured as 1.074 µm (Figure 9) while the organism grown in the presence of dye was measured to be 1.131 µm (Figure 10).

Decolorization of RBB by immobilized cells of *B. thuringiensis* BYJ1

The immobilization is confinement technique which helps to separate active biological agents from other components of reaction mixture. In bioremediation, this technique is very useful for the treatment of toxic agents. The textile effluents contain variety of dyes in very high concentrations along with other toxic chemicals. More over the pH of the effluents is also one of the factors which greatly affect the growth and stability of the organism during the treatment. The encapsulation protects organism from all above challenges during biological treatment of dye effluents.

In immobilized state, the *B. thuringiensis* BYJ1 was more efficient in decolorization of dye over the free cells. In static conditions, 99% decolorization was achieved within 72 h in immobilized state while it took 96 h in free cells condition (Figure 11).

Role of *B. thuringiensis* BYJ1 plasmid in decolorization of RBB

The ~5.5 kb plasmid was detected on agarose gel from

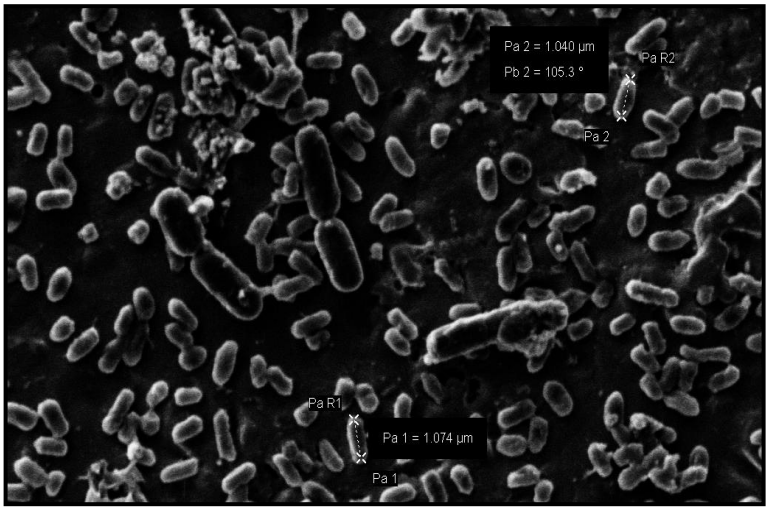

Figure 9. Scanning electron micrograph of *B. thuringiensis* BYJ1 cells grown without RBB.

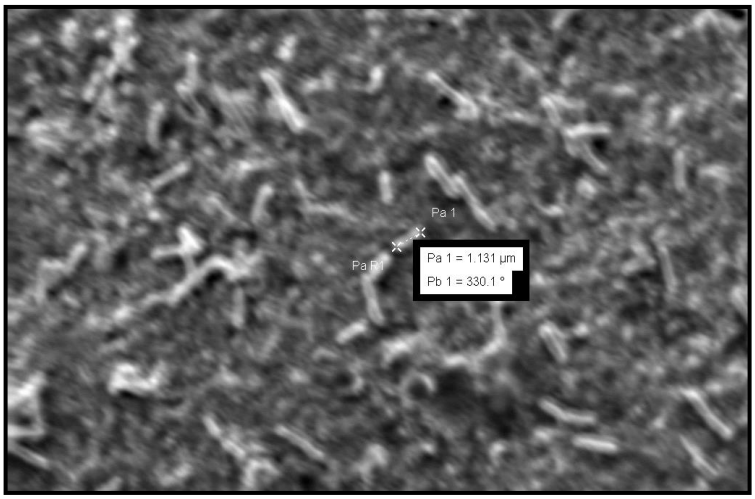

Figure 10. Scanning electron micrograph of *B. thuringiensis* BYJ1 cells grown in the presence of RBB.

B. thuringiensis BYJ1 (Figure 12; L1). During the curing process, the culture of *B. thuringiensis* BYJ1 was treated with acridine orange. There were two colonies of *B. thuringiensis* BYJ1 found on the master plate of N-agar which were absent on replica of ampicillin plate. There was no plasmid detected on agarose gel from cells of cured colonies (Figure 12; L3 and L4).

When the treated samples of Remazol Black B by *B. thuringiensis* BYJ1 and cured *B. thuringiensis* BYJ1 cultures were analyzed, there was significant difference found in decolorization of dye. This was not in accordance with the observation made by Khaled et al. (2010), indicating least role of plasmid in decolorization process. After 96 h, 92% dye was decolorized by *B. thuringiensis* BYJ1 with plasmid while 78% dye was decolorized by cured *B. thuringiensis* BYJ1 (Figure 13).

The dye decolorization efficiency of *B. thuringiensis* BYJ1 was affected due to loss of plasmid by curing experiment, but the organisms could decolorize RBB in lesser extent.

Conclusion

The reduction and degradation of azo dyes under aerobic and anaerobic conditions have been extensively studied

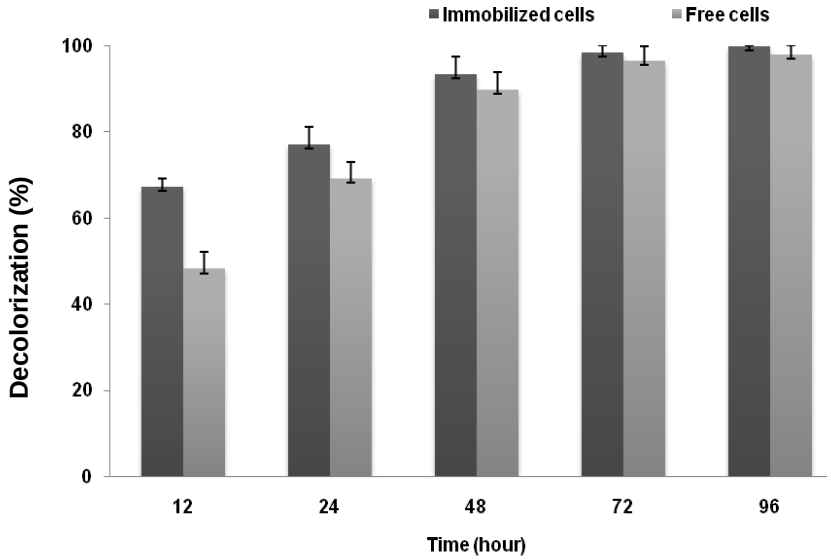

Figure 11. Decolorization of RBB by immobilized and free cells of *B. thuringiensis* BYJ1.

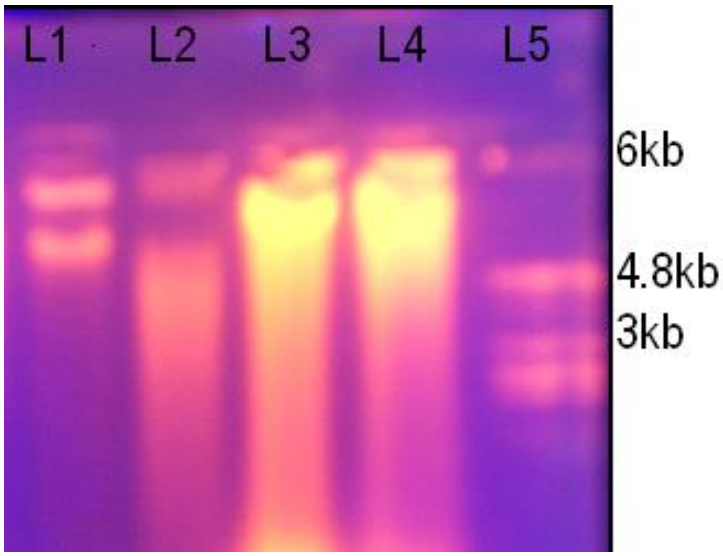

Figure 12. Agarose gel of plasmid from *B. thuringiensis* (BYJ1); L1 – BYJ1 with dye, L2 – BYJ1 without dye, L3 – BYJ1 cured colony 1, L4 – BYJ1 cured colony 2, L5 – DNA marker.

(Meiying et al., 2007). Remazol Black B is one of the difficult dyes to remove from the contaminated system. The organism *B. thuringiensis* BYJ1 was isolated from dye contaminated site and optimized for the dye degradation process. The organism could decolorizes dye in both static and shaking conditions but the static degradation products were not beneficial to plant germination while shaking dye transformed samples supported the plant growth. This was very similar to the previous studies done by Patil et al. (2008).

The soil and water samples of dye contaminated sites can provide rich source of microbes with dye tolerance and degradative potentials and such microbes and their metabolic processes can be exploited to design the technology for effective treatment of dye/textile effluents. The system using indigenous soil bacteria like

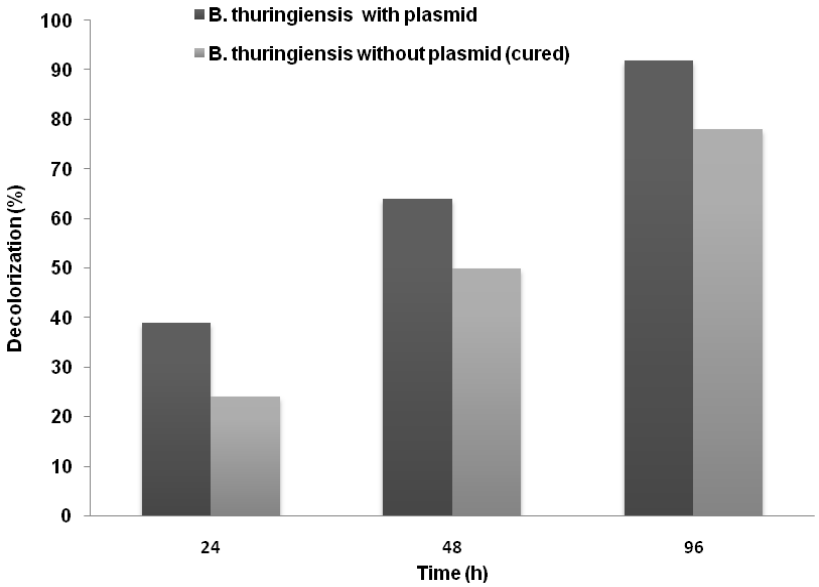

Figure 13. Effect of *B. thuringiensis* BYJ1 plasmid on decolorization of RBB.

B. thuringiensis BYJ1 can be used to design such technology which can degrade dye in shaking condition and the treated sample can be used for irrigation of agriculture fields. Application of immobilization techniques will add to the efficiency of the technology.

The *B. thuringiensis* BYJ1 was detected with a plasmid. The presence of plasmid had raised the rate of decolorization of RBB while the loss of plasmid affected the decolorization potential of *B. thuringiensis* BYJ1. The further detailed molecular study in this direction may help to detect and understand the mechanism of genes involved in decolorization of RBB.

Conflict of interest

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

ACKNOWLEDGEMENTS

The authors are thankful to Dr. P. J. Vyas (Sheth M. N. Science College, Patan), Mr. Nilesh Patel (Department of Biochemistry, AAU, Anand) and Mr. Manoj Dhujagra (Department of Biotech, JAU, Junagadh) for providing FTIR, UV-visible spectroscopic and microscopic facilities, respectively.

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