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

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

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Full Length Research Paper

Restriction enzyme mediated integration and FIV lentiviral transgenesis applied to amphibians

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Restriction enzyme mediated integration (REMI) transgenesis and lentiviral transgenesis are effective methods of introducing transgenes into the genome of frogs. One aquatic amphibian species, *Xenopus laevis*, and one land dwelling species, *Litoria caerulea*, were chosen as subjects for transgenesis. REMI produced *X. laevis* that expressed the fluorescent protein DsRed. REMI was unsuccessful in producing transgenic *Litoria.* Therefore, lentiviral transgenesis was attempted. Hatchling *Litoria* tadpoles were exposed to replication defective lentiviral particles containing the coding sequence for DsRed. Histological evaluation revealed the presence of DsRed in brain, heart, liver, kidney, and muscle tissues. Therefore, lentiviral transgenesis appears to be a viable technique for producing transgenic land-dwelling frogs.

Key words: DsRed, *Xenopus laevis*, *Litoria caerulea*, restriction enzyme mediated integration (REMI), Lentivirus.

INTRODUCTION

In recent years, the African Clawed Frog *Xenopus laevis* has become a leading model organism for transgenesis. Restriction enzyme mediated integration (REMI) of DNA into sperm nuclei, and transplantation into unfertilized eggs, is a method that has been used previously to produce GFP transgenic lines of *X. laevis* (Sakamaki et al., 2004). Although positive results have been produced using the *Xenopus* cardiac actin promoter, low expression in tissues has been a problem (Sakamaki et al., 2004). The CAG promoter is a combination of the cytomegalovirus early enhancer element and the chicken beta-actin promoter, and it drives higher expression than the *Xenopus* cardiac actin promoter (Sakamaki et al., 2004). The CAG promoter has driven transgenic expression in most *Xenopus* tissues, including germline cells (Marsh-Armstrong et al., 1999). Transgenic *Xenopus* progeny show germline transmission without the CAG promoter, but expression is mosaic (Marsh-Armstrong et al., 1999). DsRed is a 28-kDa red homologue of the fluorescent protein GFP, isolated from the *Discosoma* species of coral (Matz et al., 1999). The development of DsRed transgenic *Xenopus* would support the use of *Xenopus* as a model organism because DsRed emits in the visible light spectrum.

Although there have been several lines of transgenic *Xenopus* produced, no lines of transgenic land dwelling frogs exist. The main objective was to develop methods to generate transgenic land dwelling frogs. *Litoria caerulea* (White tree frogs) is a common land-dwelling tree frog found in Australia. Frequently found inhabiting human dwellings, *Litoria* are considered docile and have been bred in the pet trade. *Litoria* have been used to study parotoid gland secretion, natural insect repellents, lungworm infections, wet adhesion, skin water loss properties, and hind limb locomotion studies (Christian and Parry, 1997; Crockett and Peters, 2007; Pizzatto and

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Shine, 2006; Scholz et al., 2008; Smith et al., 2003; Williams et al., 2006).

MATERIALS AND METHODS

Frog husbandry methods

Adult X. laevis husbandry

Xenopus were maintained in sexed trios in two to five gallons of chlorine and chloramine free water at a room temperature of 21°C. Cage contents included PVC pipe and plastic plants for enrichment. Water was treated with 0.1 g/L Seachem equilibrium and 0.8 g/L Seachem Cichlid lake salts to bring the hardness to 215 ppm (Seachem Laboratories Madison, GA). *Xenopus* were placed on a 12/12 fluorescent lighting schedule. *Xenopus* were fed daily with *Xenopus* Express frog food (Brooksville, FL). Water changing took place once a week.

Adult Litoria caerulea husbandry

Litoria were maintained in sexed trios in 20-gallon aquariums at a room temperature of 27°C. Cage contents included a water bowl, drift wood branches, plastic foliage, with paper towel substrate. A 1 L water bowl was filled with 0.5 L sterile water. Frogs were on a 12/12 fluorescent lighting schedule. Feeding took place two days a week and consisted of four to six crickets per frog dusted with Rep-Cal Herptivite and Rep-Cal calcium with D3 (Rep-Cal, Los Gatos, CA). Water bowl and water were changed once a week when cage washing and autoclaved. Before sterilization, bowls and cages were sprayed with a 5% bleach solution. All procedures involving animals were approved by the North Carolina State University Institutional Animal Care and Use Committee.

Tadpole X. laevis husbandry

Tadpoles were first raised in an incubator with 0.1x MMR (0.01 M NaCl, 0.2 mM KCl, 0.1 mM $MgSO_4$, 0.2 mM $CaCl_2$, 0.5 mM HEPES (pH 7.8), 0.01 mM EDTA and 50 µg/mL Gentamicin) as a buffer. When tadpoles reached stage 35 of growth, they were transferred to a larger container (Nieuwkoop and Faber, 1994). Tadpoles were kept in 350 mL H_2O and fed 2-4 drops of tadpole food daily (*Xenopus* Express Brooksville, FL). Water pH was between 7.0 - 8.5 and water hardness was brought to 200 ppm. Water hardness was achieved by adding Cichlid Lake Salts at 6.4 g/L, and Equilibrium Salts at 0.8 g/L with sterile water. A Tetra Whisper aquarium bubbler was used to aerate tadpole containers (Blacksburg, VA). Room temperature was 21-24°C.

Tadpole Litoria husbandry

Litoria tadpoles were kept in groups up to seven tadpoles per liter of water. Water was treated with 0.1 g/L Seachem Equilibrium and 0.8 g/L Seachem Cichlid Lake Salts to bring the hardness to 215 ppm (Seachem Laboratories Madison, GA). Water was heated with Flex Watt Heat tape to 27°C. A Tetra Whisper aquarium bubbler was used to aerate tadpole containers (Blacksburg, VA). Tadpoles were placed on a 12/12 fluorescent lighting schedule. Tadpoles were fed daily with *Xenopus* Express Premium Tadpole Diet (Brooksville, FL). Full container cleaning took place every other day, with half of the water changed every day to remove old food. Cleaning involved rinsing the containers with water and spraying and rinsing 2 times with 70% ethanol. Bowls were sterilized by baking in a laboratory oven at 180°C overnight.

Hormone injections

X. laevis were primed with a primary intraperitoneal (IP) injection of 200 International Units of Pregnant Mare's Serum Gonadotropin (PMSG) five to seven days before induction of egg laying. To induce laying, 500 IU of human Chorionic Gonadotropin (hCG) were administered 10-12 h before procedures were scheduled to begin. As soon as frogs were injected with hCG, they were transported to the lab for observation and egg collection.

Initially the *X. laevis* hormone doses of PMSG and hCG were applied to *Litoria* but *Xenopus* doses did not induce *Litoria* egg laying. *L. caerulea* were primed with a primary IP injection of 200-300 International Units of PMSG five to seven days before induction of egg laying. To induce egg laying, *Litoria* were then given two IP injections of 250 IU of human Chorionic Gonadotropin (hCG) with 2 µg Alarelin (synthetic luteinizing hormone releasing hormone) 12 h apart. The first hCG injection occurred 12 h before the procedure was scheduled to begin.

Restriction enzyme mediated integration

Oocyte extract preparation

Oocyte extract was prepared following a published protocol detailed (Kroll and Amaya, 1996). *X. laevis* and *L. caerulea* were killed via double pithing after immersion in a 0.1% tricaine solution for 20 minor until unresponsive to needle poke. Ovaries were collected and macerated in a salt solution of 87 mM NaCa, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 5 mM Hepes, and 1% Penn-Strep pH 7.8 (OR2) with collagenase A to digest at 28°C. After washing in the salt solution, eggs were sorted in 15 mM Hepes and 50 µg/mL gentamycin (OR3), washed with OR2, and placed at 15°C overnight in OR3. Oocytes were then washed 4 times in 1X extract buffer salts (XB) and rinsed again 2 times in cytostatic factor extract buffer salts (CSF-XB) with protease inhibitors at a 1:1000 dilution. XB solution consisted of 1X XB salts, 50 mM sucrose, and 10 mM HEPES. CSF-XB solution consisted of 1X XB salts, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.7), 50 mM sucrose, and 5 mM EGTA. Protease inhibitors were 10 mg/mL Leupeptin, 10 mg/mL Chymostatin, and 10 mg/mL Pepstatin A. Samples were centrifuged for 1 min at 1000 rpm, excess buffer was removed, and solution was re-spun at 2000 rpm for 1 min or until 3 layers formed; the cytosolic layer was removed and centrifuged. The center most portion was harvested and frozen in liquid N_2.

Sperm nuclei preparation

Males were injected with 250 IU of hCG for *Xenopus* and *Litoria* an hour before killing to increase sperm production. To isolate sperm nuclei, testes were harvested and placed into a Petri dish and rinsed with 1X Marc's modified ringers solution (1X MMR) and 1X nuclear preparation buffer (1X NPB). 1X MMR consisted of 0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES (pH 7.8), and 0.1 mM EDTA. 1X NPB consisted of 250 mM sucrose, 15 mM HEPES, 0.5 mM spermidine trihydrochloride, 0.2 mM spermine tetrahydrochloride, 1 mM Dithiothreitol, and 2 mM of Ethylenediaminetetraacetic acid. Testes were then minced in 1X NPB and filtered through cheesecloth. Filtrate was centrifuged in an SS-34 rotor at 6,500 rpm (2,000 *g*) for 15 min at 4°C. *Xenopus* sperm pellets were warmed to room temperature and 50 nL of 10 mM/mL lysolecithin was added. For *Litoria,* lysolecithin alone did not subsequently de-tail the sperm; therefore an additional step of homogenizing with 100 strokes in a dounce homogenizer (Wheaton

Science Products, Millville NJ, Part # 357544) was needed before the sperm nuclei pellet was resuspended in 1X NPB/protease inhibitors/0.3% BSA. Protease inhibitors used were 10 mg/mL Leupeptin, 10 mg/mL Chymostatin, and 10 mg/mL Pepstatin A. Samples were centrifuged, and suspended in cold sperm storage buffer and frozen in liquid nitrogen in 20 µL aliquots. Sperm nuclei storage buffer consisted of glycerol, 0.3% BSA, in 1X NPB. Sperm samples were stored at -80°C. Nuclei quality was evaluated by mixing sperm nuclei with oocyte extract. Only nuclear preparation that exhibited visible sperm nuclei swelling were used for injection.

De-jelly and post de-jelly solutions for transgenesis

For *Xenopus*, a 15% sodium borate (Sb) buffer (10 mM NaOH pH to 8.5 with H_3BO_3), 2.5% cysteine free base pH 8.5 solution was used to de-jelly the eggs (Kroll and Amaya, 1996). To remove de-jelly solution, eggs were rinsed 3 times in a solution of 0.5% bovine serum albumin (BSA) in 1x MMR.

Xenopus de-jelly solution was originally applied to *Litoria*, but the *Litoria* eggs are more delicate than the *Xenopus* eggs; they exhibited visual dehydration, and embryos did not develop. A final solution of 5% Cysteine free base solution in 0.1X MMR, pH 8.5 was determined as an appropriate de-jelly solution. To remove de-jelly solution; eggs were rinsed three times with a solution of 1% BSA in 0.4X MMR.

Attempts at injecting non-dejellied eggs involved laying a 1000 µm mesh over the groups of eggs and injecting through the mesh using the same needle sizes and injection medias as with the de-jellied eggs.

Injection Media and Post- Injection medias

Xenopus eggs were injected in a solution of 6% Ficoll 400 and 0.5% BSA in 0.4X MMR. Following injection, *Xenopus* eggs were placed in a solution of 6% Ficoll 400 and gentamycin (50 µg/mL) in 0.1X MMR.

Due to the *Litoria* eggs failure to activate when the *Xenopus* injection solutions were used, the solutions were adjusted. *Litoria* eggs were injected in a solution of 20X MMR, Ficoll 400, and 10% BSA. Following injection *Litoria* eggs were placed in a solution of 0.4X MMR, 2% BSA medium with 50 µg/mL gentamycin.

REMI egg injection procedure

The pCAG DsRed vector was chosen for transgenesis (Cambridge MA). Restriction enzyme SpeI and ApaLI digests were performed on the pCAG vector. SpeI cut the plasmid once at 18 bp; ApaLI cut the plasmid twice, at 4,010 and at 5,255 bp. The linearized plasmid contained an intact promoter and DsRed gene after restriction enzyme digest. Restriction enzymes were inactivated by placement in 4°C overnight. Sperm nuclei solutions used in nuclear micro injection to produce DsRed transgenic *Xenopus* and *Litoria* consisted of: sperm nuclei (50 sperm nuclei/nL), ApaLI or SpeI digested p-CAG DsRed plasmid, 0.5 IU of ApaLI or SpeI, oocyte extract, sperm dilution buffer, $CaCl_2$ and $MgCl_2$. After de-jellying, eggs were rinsed 3 times with post de-jelly rinse and loaded into a Petri dish of injection media. Oocyte extract and sperm mix were combined and loaded into a 100 µm beveled needle. Eggs were injected with 4-5 nL of sperm nuclei solution at a rate of 50 µL/hr. Sperm solutions were used up to 30 minutes after thawing.

An unpaired student t-test was applied to the population data.

Visual screening

DsRed positive tadpoles were determined by observation using epifluorescence illumination through a Texas Red filter set at 20x magnification with a LEICA DM IRB microscope (Richmond, Illinois). Pictures were taken with an RT slider Spot camera (Sterling Heights, Michigan).

Lentiviral transgenesis

A second protocol was developed to incubate hatched *Litoria* tadpoles in a solution containing lentivirus overnight to generate transgenic tadpoles. Hormone injections and timing were identical as used to stimulate sperm production and egg laying in the REMI protocol. To induce breeding, a rain chamber was constructed from a 20-gallon aquarium, which was filled halfway with water containing 0.1 g/L Seachem Equilibrium and 0.8 g/L Seachem Cichlid Lake Salts. The water had hardness (GH or general hardness) of 215 ppm heated to 27°C, and pumped into a container above the aquarium. Water was allowed to drip onto driftwood, a platform, and plastic plants to mimic the wet breeding season. Following PMSG injection, one to two female *Litoria* were placed into a rain chamber with three to six males. *Litoria* remained in the rain chamber for seven days or until eggs were laid. If eggs were laid, the adult *Litoria* were removed and eggs were allowed to hatch in the rain chamber.

Lentivirus production

The lentiviral vector system chosen for transgenesis was pCDF1 (System Biosciences, Mountain View. CA) a derivative of Feline Immunodeficiency Virus (FIV) requiring packaging plasmids for viral production (System Biosciences, Mountain View, CA). The gene isolated for transgenesis was DsRed from the plasmid pCAG, driven by the chicken beta actin promoter. A replication defective lentivirus was generated using the PEG-it kit (System Biosciences, Mountain View, CA). The DsRed construct was ligated into pCDF1. Subsequently, the plasmids were transfected into HEK 293 cells, which secreted the replication defective lentiviral particles into the cell culture media. Viral pseudoparticles were collected, frozen, and titered using NIH 3T3 cells. Cultures exhibited titers averaging $1.6x10^6$ TU/mL.

Lentiviral infection media

Three basal medias were chosen to select for lentiviral media infectivity: Dulbecco's Modified Eagle Medium (DMEM), NCTC 109, and Leibovitz 15 (L15) medium. NIH 3T3 cells were plated at $1x10^6$ cells/mL in three wells of three 6-well plates in 3 mL of media with 1 µL/mL media of [100 µg/mL] polybrene. A day later cells were infected with replication defective lentiviral particles and observed for fluorescence three days later. Pictures were taken of the wells, and infected versus non-infected cell populations were recorded. Transformation units were determined between infected and non-infected cell population data (Table 4). Standard error was calculated and propagated for each well accordingly to give a total standard error for the media. A one-way analysis of variance (ANOVA) evaluation was performed on the infection populations to determine whether the medias caused the difference in infection rates. Means were separated using least significant difference (LSD) between each media.

Lentivirus incubated tadpole method

Immediately after tadpoles hatched, they were removed from the rain chamber and transported to the laboratory. Procedures were performed under a class II Biosafety Cabinet. Tadpoles were

Table 1. REMI production of transgenic *Xenopus laevis* categorized by restriction enzymes SpeI and ApaLI used on pCAG-DsRed.

Parameter	Restriction Enzyme SpeI	Restriction Enzyme ApaLI
Fluorescent tadpoles	88	49
Total tadpoles	392	433
Well formed tadpoles	129	217
Percent fluorescent	22%	11%

placed into 250 mL beakers with 20 tadpoles per beaker. As a media for the virus and the tadpoles, NCTC 109 media was diluted in half with sterilized water to a volume of 40 mL. To create frog water, 0.1 g/L Seachem Equilibrium and 0.8 g/L Seachem Cichlid Lake Salts was mixed in sterile water. The water had a hardness (GH or general hardness) of 215 ppm. To assist in transduction of viral particles into tadpole cells, 64 µL polybrene (100 µg/mL) was added to the solution. Lastly, 40 mL of virus was added to the solution, making the final ratios half virus, half frog water/NCTC 109 media/polybrene (50 µg/mL). Tadpoles were incubated in virus for up to 12 h. Solutions with tadpoles were directly aerated. Tadpoles were observed every 15 min.

After incubating in the virus for 12 h, the tadpoles were moved to containers of the same size with water. When 20 min had passed, the tadpoles were moved to their final destination of 5 large 1.5 liter glass Pyrex bowls. Half of the tadpole water was changed every other weekday with a full change once a week. Tadpoles were cultivated until fully metamorphosed (typically after one to two months); samples of water were incubated with NIH 3T3 cells to ensure tadpoles were not shedding any retrovirus. No DsRed positive cells were observed.

Litoria histological evaluation

Histological evaluations were performed on 6 incubated *L. caerulea* tadpole tissues. *Litoria* samples were placed in an 80% PBS, and sucrose solution. Samples were stored over night at 4°C. A 2:1 solution of 20% sucrose OCT media was prepared as an embedding medium (Tissue Tek Sakura, Alphen aan den Rijn, Netherlands). *Litoria* were placed into dry embedding molds, which were filled with embedding medium. The mold was then taken and placed in a small container of methyl butane and floated over liquid nitrogen until the embedding media solidified. Ten micron thick sections were placed on glass slides and mounted in a glycerol based media containing 1 mg/mL p-phenylenediamine (Swartz et al., 1990). Cover slips were sealed with clear nail polish and samples were observed under a LEICA DM IRB microscope (Buffalo Grove, IL).

RESULTS

X. laevis REMI restriction enzyme results

The hatchability of REMI transgenic *Xenopus* tadpoles are listed in Table 1. Out of the 825 total tadpoles that hatched, 346 hatched well formed, and 137 expressed the DsRed protein. The total percent of tadpoles that visually expressed the DsRed protein were 22% of the tadpoles produced using SpeI, and 11% using ApaLI. Statistical analysis of an unpaired student t-test was performed on the complete hatchability data, but no significant difference was found.

Pictures of DsRed-positive developing embryos and hatchlings were taken under incandescent illumination. Under incandescent illumination developing embryos and hatchlings display pink areas. (Figures 1 and 2).

REMI De-jelly results

Several de-jelly solutions were applied to *Litoria* eggs to adjust for dehydration (Table 2). A de-jelly solution of 0.5% BSA and 3%-6% Ficoll in 0.4% MMR was found to qualitatively dehydrate *Litoria* eggs the least. After an adequate de-jelly solution was found, injection solutions were also varied using the optimal de-jelly solutions (Table 3). A solution of 6% Ficoll and 0.5% BSA in 0.4X MMR was found to assist in activating *Litoria* eggs. As a control injection through the jelly-coat was also attempted on *Xenopus* and *Litoria*. Mesh was used to hold the eggs in place and create a single layer of eggs for injection. Injection through the mesh and jelly coat proved difficult; the mesh obscured the location of the poles of the eggs for correct injection site and the eggs would move easily and tear when pierced with the needle. A second problem encountered with this method was the injection media used typically failed at activating the eggs. As a result few tadpoles were produced and it was determined de-jellying was an easier and more efficient method. The REMI method failed to produce embryo development past neural tube formation in the *Litoria* species, which may be related to tadpole management.

Lentiviral infection media

Cells were plated at $1x10^6$ cells/mL per well. Total cell numbers for ten picture sample of the wells were as follows: 31,118 total cells for DMEM, 4,786 total cells for NCTC 109, and 3,937 for L15 media. For three media wells, the average transformation units were as follows; For DMEM media $8.29x10^2$ TU/mL, $1.16x10^5$ TU/mL for NCTC 109 media, and $4.39x10^4$ TU/mL for L15 media were counted (Table 4). A one way Analysis of Variance ANOVA was performed to determine whether there was a significant difference of infection rate among medias:

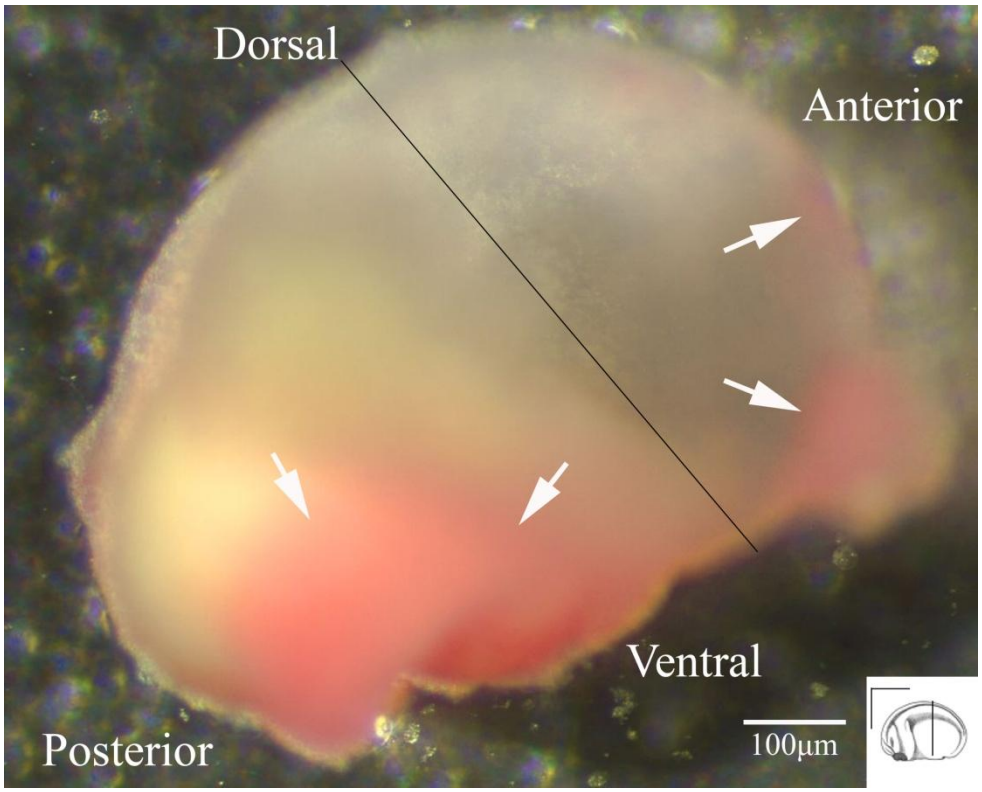

Figure 1. Stage 20 DsRed positive *Xenopus* embryo. Positive areas (pink) observed were of the embryos ventral hemisphere (denoted by arrows).

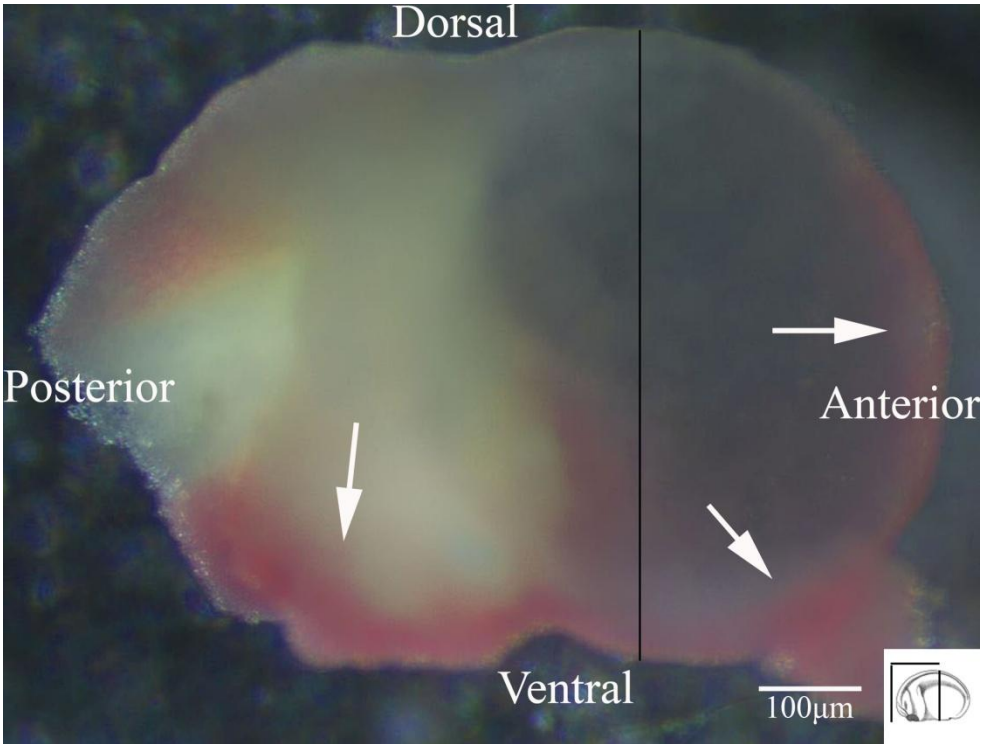

Figure 2. Stage 20 DsRed positive *Xenopus* embryo. Positive areas (pink) observed were of the embryos ventral hemisphere (denoted by arrows).

Table 2. REMI de-jelly solutions for *Litoria caerulea* and *Xenopus laevis* varied to decrease *Litoria caerulea* egg dehydration.

Species	De-jelly Solutions	De-jelly Results
Litoria caerulea	None- Injection through jelly coat	-Eggs were difficult to inject, none developed
	1 mg/mL Hyaluronidase in 1X MMR	-Eggs dehydrated and not de-jellied
	10% Cysteine free base in sterile water	-Eggs dehydrated and partially de-jellied
	2.5% Cysteine free base in 15% Sb	-Eggs dehydrated and de-jellied
	5% Cysteine free base in 1X MMR	-Eggs dehydrated the least and de-jellied
		-Well established method
Xenopus laevis	2.5% Cysteine free base in 15% sodium borate (Sb) buffer (10mM NaOH pH to 8.5 with H_3BO_3)	-Eggs dehydrated the least
		-Majority of transgenic tadpoles produced
		-Eggs did not activate well/method was difficult
	None- Injection through the jelly coat	-2 tadpoles/100 intact vs.
		6 tadpoles /100 de-jellied

Table 3. REMI injection solutions for *Litoria caerulea* varied to increase egg activation.

Specie	Injection Media	Injection Results
Litoria caerulea	6% Ficoll in 0.1X MMR	Eggs dehydrated, No egg activation
	6% Ficoll in 0.4X MMR	Eggs dehydrated, No egg activation
	0.5% BSA and 6% Ficoll in 0.4X MMR	Eggs dehydrated, Egg activation

Table 4. Lentiviral titration of 3T3 cells in three medias to determine viral media preference.

Media	TU/mL	Standard Error
DMEM	$8.29x10^{2\ c}$	$4x10^{2}$
NCTC 109	$1.16x10^{5\ ab}$	$4x10^{4}$
L15	$4.39x10^{4\ b}$	$1x10^{4}$

ANOVA: Df= 2; P-Value= 0.0069193. Values with different superscript are significantly different (P<0.05).

there was significant difference found between DMEM and the other medias, but there is no significant difference between the means of NCTC 109 and L15 (Statistical Methods Table 4). To confirm the possibility of *Xenopus/amphibian* cell infectivity, embryo cells were cultured and transfected with DsRed (Figure 3). Positive cells exhibited fluorescence.

Lentiviral *L. Caerulea* microscopy

Transgenic *Litoria* tadpoles were successfully generated, cultivated, killed and observed. Samples were collected from transgenic and control froglets one to two months old. Tissue sections from control and treated tadpoles were examined for DsRed fluorescence under a epifluorescence illumination (Figures 4, 5, 6, and 7). Tissues observed to be positive for DsRed expression were brain, heart, muscle, and kidney.

DISCUSSION

Restriction enzyme mediated integration (REMI) successfully produced transgenic *X. laevis*. After statistical analysis using an unpaired student t-test, it was found that SpeI and ApaLI are both valid restriction enzymes to use in REMI. Because of the success in the *Xenopus,* the REMI protocol was attempted with *Litoria*. The *Xenopus* hormone injection failed to produce egg laying in the *Litoria*. PMSG was used as hormonal “priming” because it has been shown to increase the total number of early developing oocytes in the ovaries of *Rana cyanophlyctis* without increasing the number of mature oocytes (Pancharatna and Saidapur, 1984). Induction was first attempted in *L. caerulea* using a single dose of 1 mg/kg LHRH, which previously induced spermatozoa production in the tree frog *Hyla regilla,* with no success (Licht, 1974). It is likely that a single injection of LHRH did not induce egg laying in the *Litoria* because its effects are similar to PMSG; increasing the number of early stage oocytes without increasing maturation or ovulation. A second attempt using PMSG as a hormonal primer and a combination of

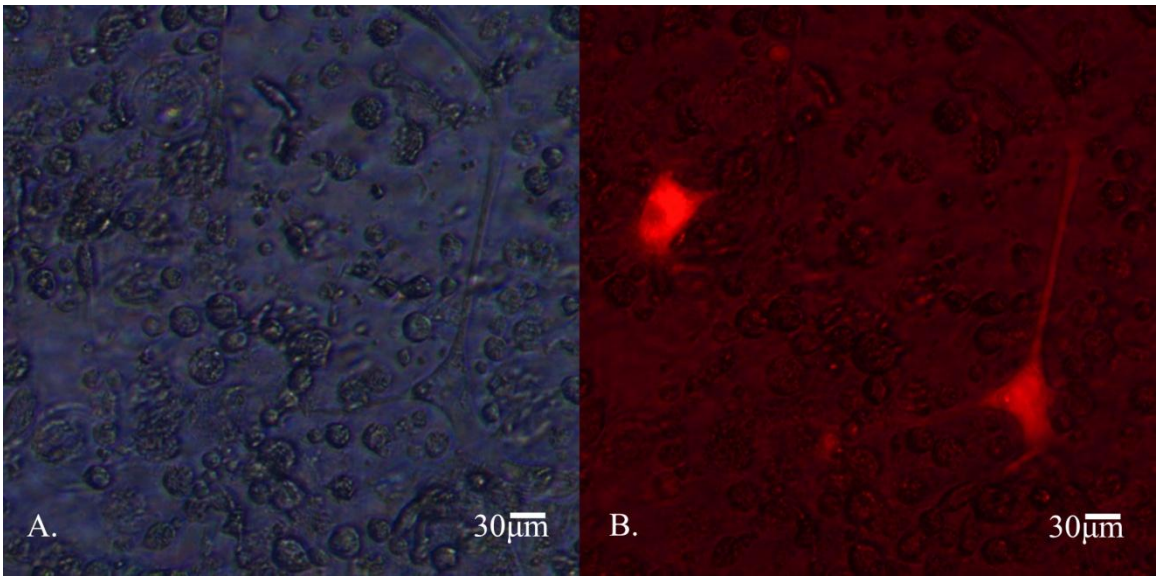

Figure 3. Brightfield (A) and epi-fluorescent (B) illumination of cultured DsRed *Xenopus laevis* tadpole cells that were transfected with DsRed. B. Fluorescence (Red) indicates positive results. Scale bar is 30 µm.

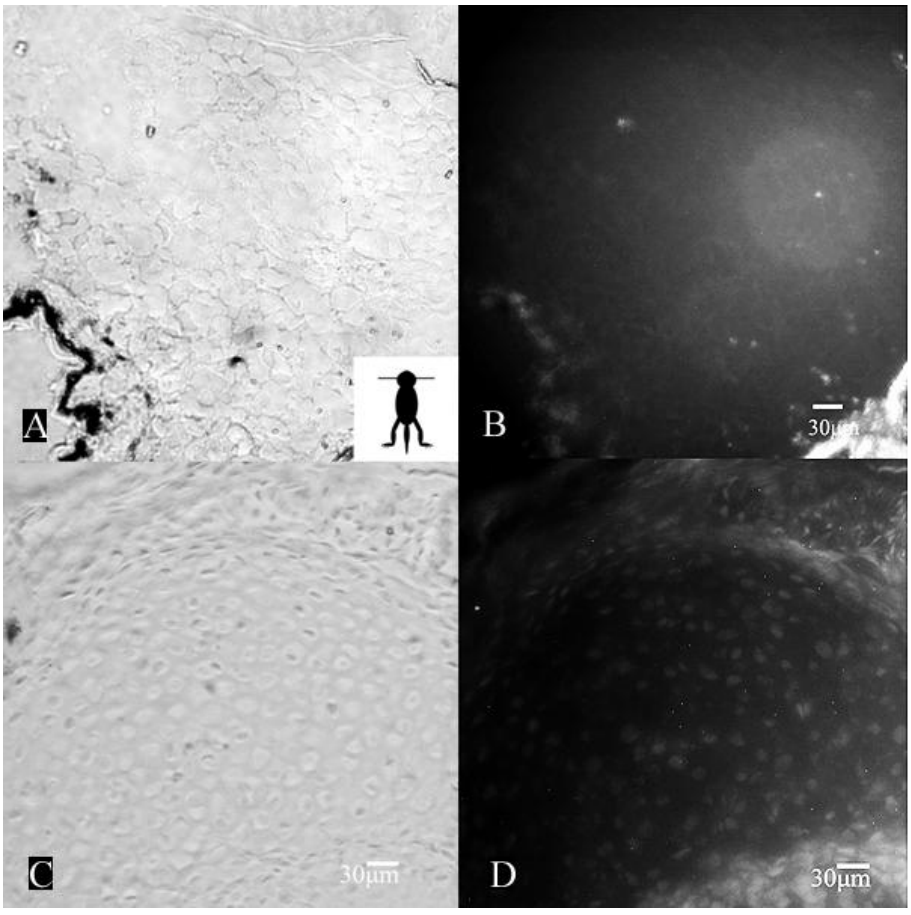

Figure 4. Tissue sections from untreated *Litoria* tadpoles (A, B) and *Litoria* tadpoles treated with cell culture supernatant containing lentiviral particles encoding Ds Red (C, D). Schematic inset of frog shown within panel A illustrates the plane of tissue section from the whole animal Images illustrate neural tissue. Sections are shown under bright field (A, C) and epifluorescence illumination (B, D). Scale bar is 30 microns.

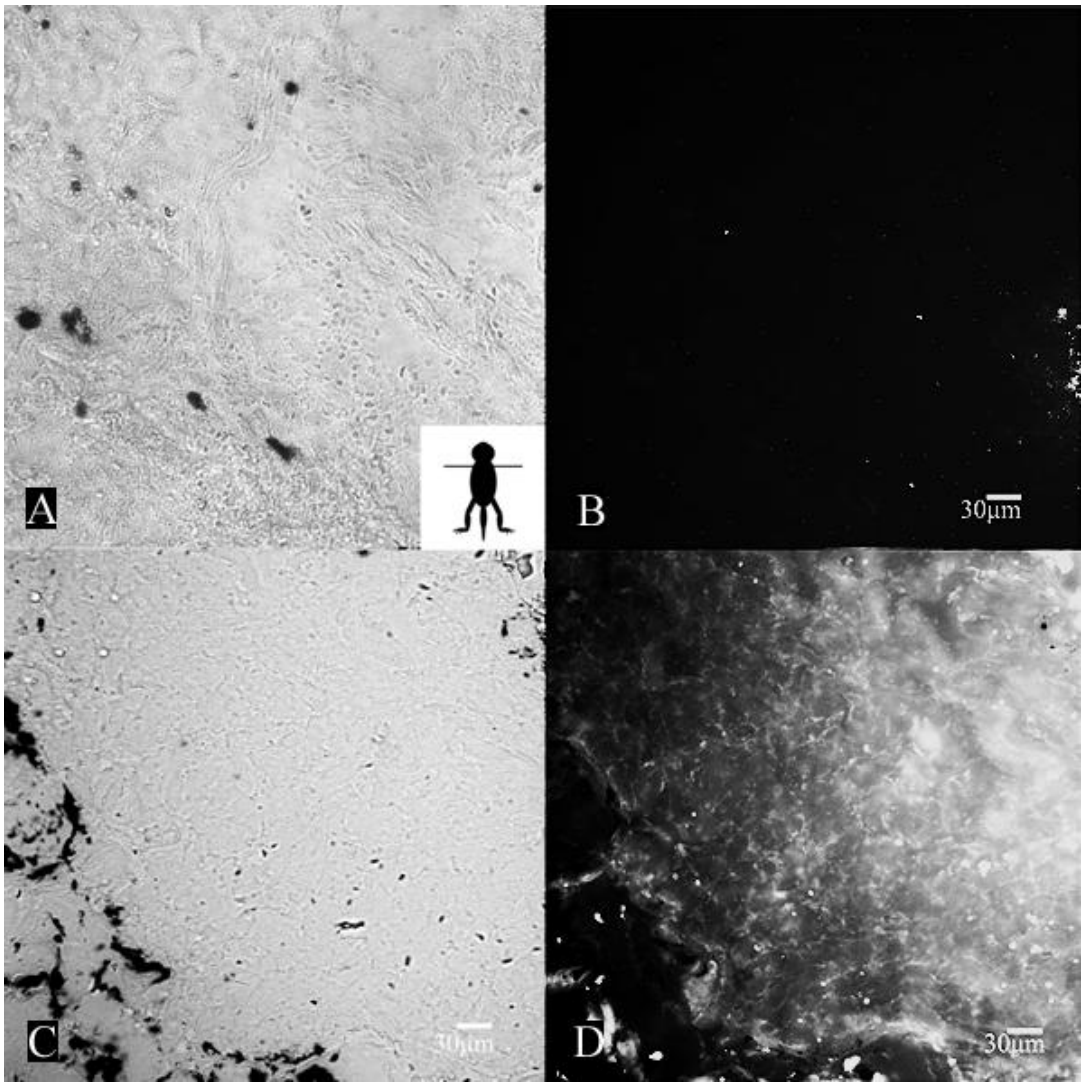

Figure 5. Tissue sections from untreated *Litoria* tadpoles (A, B) and *Litoria* tadpoles treated with cell culture supernatant containing lentiviral particles encoding Ds Red (C, D). Schematic inset of frog shown within panel A illustrates the plane of tissue section from the whole animal Images illustrate heart tissue. Sections are shown under brightfield (A, C) and epifluorescennce illumination (B, D). Scale bar is 30 microns.

hCG and Alarelin peptide hormone (synthetic LHRH) as an inducer, according to a protocol used to induce *Bufo baxteri,* was successful inducing consistent egg laying (Browne et al., 2000). *Litoria* were given hCG as an inducer because it has been shown to shorten oocyte maturation and stimulates ovulation in *X. laevis* (Browne et al., 2000).

REMI was attempted with the jelly coat intact, but the injection proved difficult; lower egg activation was observed and proper placement of the injection site was obscured because of the need for mesh to create a single layer of cells. Due to these problems, the de-jelly method was viewed as a more efficient method of production. Several de-jelly and injection medias were attempted to decrease the dehydration of *Litoria* eggs. Solutions containing cysteine free-base appeared to be the most successful in de-jellying the eggs. A higher concentration of cysteine was required to de-jelly *Litoria* eggs than *Xenopus*. These differences contributed to why REMI failed to produce transgenic *Litoria*. Failure was likely due to a difference between the *Xenopus*, and *Litoria's* tolerance to de-jellying the eggs. The jelly coat's function is to allow attachment to vegetation, provide protection, assist in sperm binding, prevent polyspermy, and act as a barrier to prevent embryo anoxia (Seymour and Bradford, 1995). With removal of the jelly coat, the embryo is more vulnerable to environmental influences.

It has been shown that egg quality and survivability depend on several factors including husbandry conditions of the adult females, water salinity, water contaminants (chlo-

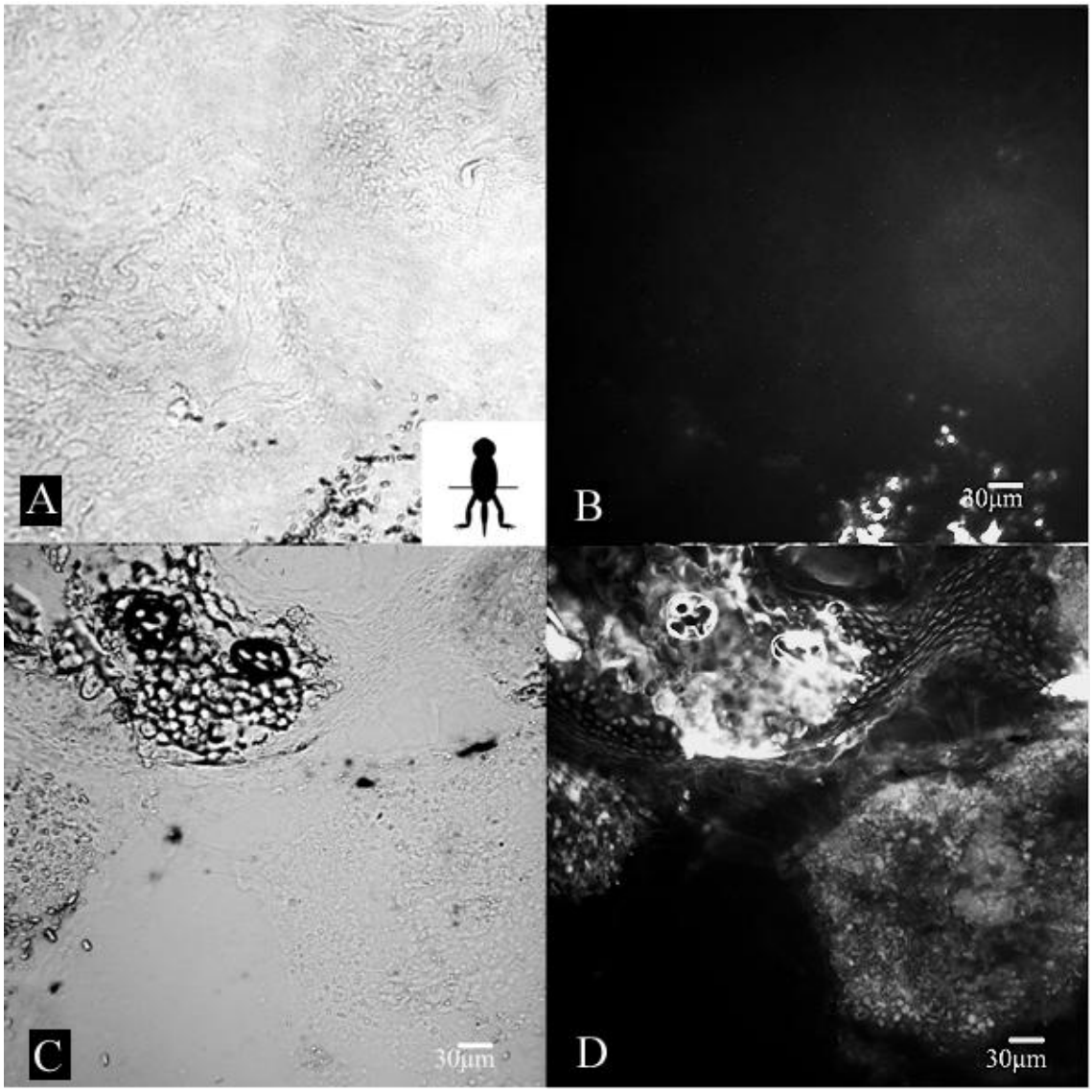

Figure 6. Tissue sections from untreated *Litoria* tadpoles (A, B) and *Litoria* tadpoles treated with cell culture supernatant containing lentiviral particles encoding Ds Red (C, D). Schematic inset of frog shown within panel A illustrates the plane of tissue section from the whole animal Images illustrate kidney tissue. Sections are shown under brightfield (A, C) and epifluorescennce illumination (B, D). Scale bar is 30 microns.

rine, ammonia, nitrate, and nitrite), and general hardness (GH) of the water; with hardness of water and presence of calcium and magnesium ions being more important than alkalinity or GH (Godfrey and Sanders, 2004). When *Xenopus* eggs were of poor quality, an increase in general hardness increased overall firmness of the vitelline membrane and normal development and survivability of embryos increased (Godfrey and Sanders, 2004). The protocol for maintaining transgenic embryos was modified from the *Xenopus* husbandry protocol to adjust for embryo fragility after de-jellying with an increase in GH. The de-jellying process may affect vitelline membrane elasticity. The vitelline membrane plays a significant role in osmoregulation of the developing embryo (Mild et al., 1973). During the de-jellying process, it is possible the vitelline membrane of the *Litoria* eggs was damaged and the general hardness for the de-jellied egg medias needed to be increased further. It is possible that negative results were produced because the husbandry for the *Litoria* tadpoles needed to be adjusted further to account for different salinity, GH, and aeration.

It is also possible during the injection procedure more than one sperm nuclei were injected in some eggs producing abnormal embryo growth. In previous studies, it has been shown that aquatic frogs produce smaller ovum size than the terrestrial species allowing for more oxygennation to the embryo, which assists in faster development (Seymour, 1999). Removal of the jelly coat would influence

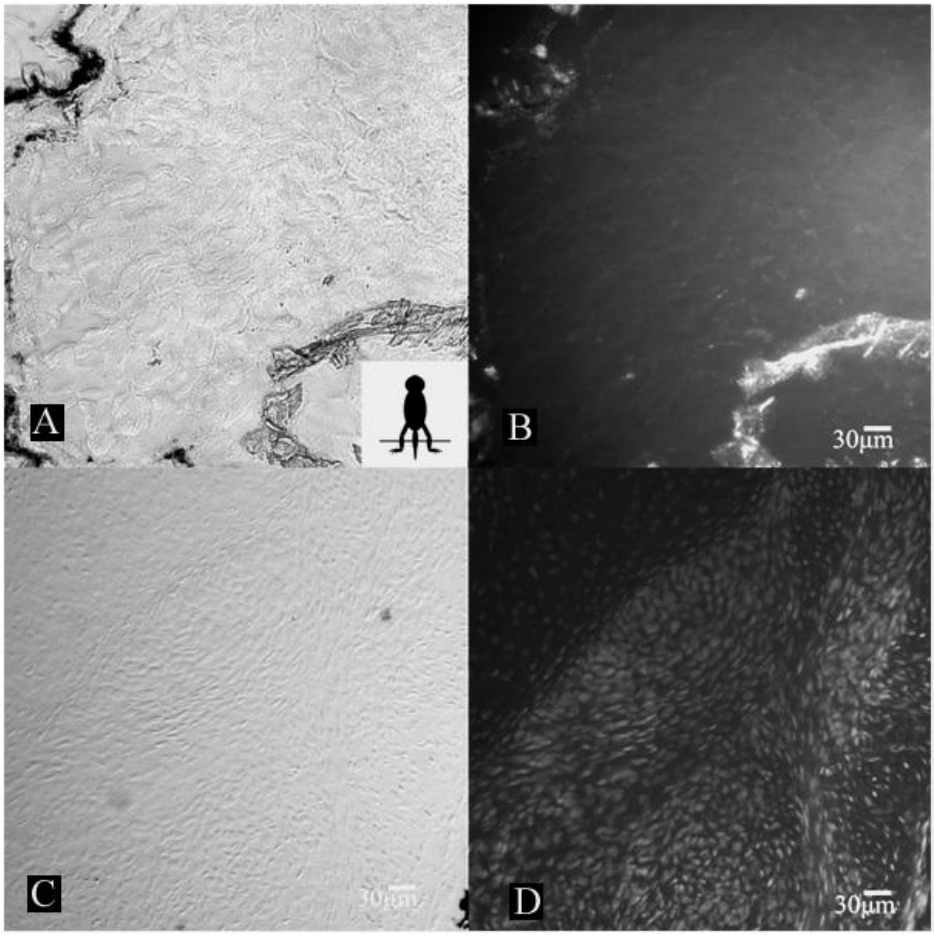

Figure 7. Tissue sections from untreated *Litoria* tadpoles (A, B) and *Litoria* tadpoles treated with cell culture supernatant containing lentiviral particles encoding Ds Red (C, D). Schematic inset of frog shown within panel A illustrates the plane of tissue section from the whole animal Images illustrate muscle tissue. Sections are shown under brightfield (A, C) and epifluorescennce illumination (B, D). Scale bar is 30 microns.

the embryos ability to respire and could account for slower development/embryo death due to inadequate oxygen/carbon dioxide regulation in the terrestrial species. Although unlikely, polyspermy causes abnormal development in monospermy species.

Transposon transgenesis is a common method used in biotechnology to create transgenic *Drosphilia, C. elegans*, and plants (Grabher and Wittbrodt, 2009). The process is similar to REMI involving injection of transposon DNA with synthetic transposase mRNA to cause random insertion into the host genome. Transposon technology does not require a viral backbone like lentivirus transgenesis; the transposon itself is a naturally occurring mobile DNA element (Yergeau et al., 2010). Recently, this technology has been applied to vertebrate species, most notably *X. laevis* (Grabher and Wittbrodt, 2009). A major disadvantage to using transposon transgenesis is that its efficiency is inversely related to the inserted transposon's size, making it an inefficient candidate for large gene insertion (Grabher and Wittbrodt, 2009). Transposon transgenesis also requires species-specific cofactors limiting its application to thoroughly studied species (Grabher and Wittbrodt, 2009). Mosaic expression is common due to the process requiring coinjection of transposase mRNA to catalyze the insertion reaction; the transposase mRNA may not be transcribed before the embryo completes its early stages, meaning the transposase enzyme will not be available to catalyze the reaction in those cells (Yergeau et al., 2010). For these reasons, transposon transgenesis was viewed as an inadequate method for this study.

Lentiviral transgenesis produced DsRed positive *L. caerulea* tadpoles. Lentiviral transgensis only results in stable gene insertion into the genome (Cockrell and Kafry, 2007). The lentivirus vector pCDF1 was driven by the CMV/chicken beta-actin promoter, which drives protein production in the skin, liver, brain, heart, kidney, spleen and lung tissues (Fahim et al., 2009; Lois et al., 2002). Expression was visually confirmed in transgenic tadpole and frog tissues of the brain, heart, kidney, liver,

and muscle cells. The distribution of DsRed expression is similar to studies of GFP lentiviral transgenic mice, where the expression of transgene proteins was found in all tissues and organs analyzed including, skin, bone, muscle, lung, liver, stomach, intestine, kidney, brain, retina and gonads (Lois et al., 2002; Wiznerowicz and Trono, 2005).

Two transgenic species of amphibians were created using the methods of REMI and lentiviral transgenesis. Individuals in the *Xenopus* and *Litoria* species reach maturity at 1-1.5 years of age. Although germline transmission to the F1 generation was beyond the scope of the current study because of the long interval between generations, it is likely that successful germline transmission will occur, as evidenced by the previous success found in chickens, mice, and GFP transgenic *Xenopus laevis* (Lois et al., 2002; Marsh-Armstrong et al., 1999; Mozdziak et al., 2005).

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Full Length Research Paper

The antibacterial effect of phyto-mediated silver nanoparticles produced from *Ocimum sanctum* L. (Lamiaceae) leaf extract on textile fabrics

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The *Ocimum sanctum* (holy thulsi) leaves extract perform as a reducing and capping agent in the formation of silver nanoparticles. A UV-VIS spectrum of the silver nanoparticles showed a peak at 421 nm. The morphology and size of the silver nanoparticles were carried out by the transmission electron microscope (TEM) and scanning electron microscope (SEM). The sizes of the synthesized silver nanoparticles were found to be in the range of 7 to 45 nm. The structural characteristics of biomolecules hosted silver nanoparticles were studied by X-ray diffraction. The chemical composition of elements present in the solution was determined by energy dispersive spectrum. The FTIR analysis of the nanoparticles indicated the presence of proteins, which may be acting as capping agents around the silver nanoparticles. This study reports that green synthesis is medicinally useful nanoparticles to avoid toxic chemicals with adverse effects in medical applications rather than physical and chemical methods. The antibacterial activity of phyto mediated silver nanoparticles was assessed by the paper disc method against *Klebsiella pneumonia* and 12 mm clear zone was observed. The antibacterial activity of the finished fabrics was assessed quantitatively by reduction test. The topographical analysis of the treated fabric and untreated fabric was studied and compared. The results show that the finished fabric demonstrated significant antibacterial activity against *Escherichia coli.*

Key words: Silver nanoparticles, antimicrobial activity, cotton, green synthesis.

INTRODUCTION

Remarkable advances are made in the field of nanobiotechnology to harness the benefit of life sciences (Huang et al., 2007), healthcare (Ahmad et al., 2010) and Industrial biotechnology (Elechiguerra et al., 2005). Nanomaterials especially silver nanoparticles may provide solutions to technological and environmental challenges in the areas of solar energy conversion, catalysis, medicine, nano-biosensor, targeted drug delivery system and water treatment (Bao et al., 2007). Silver has long recognized as an inhibitory effect on microbes present in medical and industrial process (Reda et al., 2011) and have antimicrobial properties with low toxicity (Jain et al., 2009).

This increasing demand must be accompanied by "green" synthesis procedures. A number of approaches are existing by the use of chemical and photochemical reactions for syntheses of silver nanoparticles such as reverse micelles, thermal decomposition of silver compounds, radiation assisted, electrochemical, sonochemical and microwave assisted process (Parashar et al., 2009). Chemical synthesis methods lead to presence of toxic chemical engrossed on the surface that nanoparticles may have adverse consequence in the medical applications (Jain et al., 2009).

Now a day biosynthesis of nanoparticles are used by most researchers to overcome the above problems (Udayasoorian et al., 2011). The green synthesis is more

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advantageous over chemical and physical method as it is cost effective and environment friendly (Kowshik et al., 2003; Nabhikha et al., 2009).

Concerning the biological application of nanoparticles, it has been emphasized that methods of synthesis through biological systems namely; microorganisms including bacteria, yeasts, fungi and diatoms synthesizing inorganic materials either intra or extracellular would make the nanoparticles more biocompatible (Thirumurugan et al., 2011). One of them is the synthesis using plant extracts eliminating the elaborate process of maintaining the microbial culture and often found to be kinetically favorable than other bioprocesses. Bio-molecules as reducing agents are found to have a significant advantage over their counterparts as protecting agents.

With the growth in world population and the spread of disease, the number of antibiotic resistant microorganisms is rising along with the occurrence of infections from these microorganisms. With this increase in health awareness, many people focused their attention on educating and protecting themselves against harmful pathogens. It soon became more important for antimicrobially finished textiles to protect the wearer from bacteria than it was to simply protect the garment from fiber degradation (Rajendran et al., 2010).

In these aspects synthetic methods based on naturally occurring biomaterials provide an alternative means for obtaining these nanoparticles. During recent times several groups have achieved success in the synthesis of Ag nanoparticles using extracts obtained from unicellular organisms like bacteria and fungi as well as extracts from plant parts, for example, geranium leaves, lemon grass, neem leaves, Aloe vera, and several others (Ahmad et al., 2003; Nair and Pradeep, 2002; Shahverdi et al., 2007; Ahmad et al., 2010; Shiv Shankar et al., 2005; Chandran et al., 2006). The spectacular success in this field has opened up the prospect of developing bio-inspired methods of synthesis of metal nanoparticles with tailor-made structural properties. Among the various bioreductants, *Ocimum sanctum* (Labiatae), a sweet basil leaf extract was chosen for the present study since they have essential oils and synthesized Ag nanoparticles are used for preparation of antimicrobial fabrics.

MATERIALS AND METHODS

Chemicals

All analytical reagents and media components were purchased from Hi-Media (India).

Collection of plant leaves and preparation of extracts

O. *sanctum* plant leaves were collected from a university campus itself and thoroughly washed with distilled water and kept it at room temperature. The plant leaf broth solution was prepared by taking 20 g of leaves, cut into small pieces and ground in a mortar and pestle with 100 ml of sterile distilled water and centrifuged at 3000 RPM to get the leaf extract.

Synthesis of silver nanoparticles

Two flasks were taken, in the first flask 25 mL of leaf broth was added to 200 ml of 1 mM aqueous $AgNO_3$ solution for reduction of Ag^+ ions and no leaf broth was added to the second flask and it considered as control. Both flasks were kept at room temperature on shaker for 24 h.

Extraction of silver nanoparticles

After the incubation period the silver nanoparticle solution thus obtained was purified by repeated centrifugation at 15,000 rpm for 20 min followed by re-dispersion of the pellet in deionized water.

Ultraviolet-visible spectroscopy (UV-VIS) spectra analysis

UV-Vis spectroscopy measurements (Shimadzu, Japan) were carried out as a function of time of the reaction at room temperature operated at a resolution of 1 nm. The reduction of silver ions was confirmed by qualitative testing of supernatant obtained after centrifugation with a pinch of NaCl. The reduction of silver ions was monitored by measuring the absorbance of the reaction mixture in a range of wavelength from 200 to 800 nm to find the absorbance peak different intervals (0, 30 and 60; 2, 4, 8, 16 and 24 h).

Fourier transform infrared (FTIR) analysis

For Fourier transform infrared (FTIR) spectroscopy measurement, the following method was adopted. The silver nanoparticles were synthesized after 24 h of incubation with the leaf extracts, centrifuged at 10,000 rpm for 15 min, and their pellets were then dried and the powder was subjected to FTIR spectroscopy measurement (Perkin Elmer spectrophotometer) in the reflectance mode at a resolution of 4 cm^{-1} in KBr pellets.

Scanning electron microscopy and energy dispersive spectroscopy (EDS) analysis

After 24 h of incubation leaf extracts were analyzed under a scanning electron microscopic (JOEL) at a voltage of 120 kV. EDS analysis was carried out on the JEOL Analysis Station at an accelerating voltage of 20 keV.

Transmission electron analysis

Samples for transmission electron microscopy (TEM) analysis were prepared by drop coating biologically synthesized silver nanoparticles solution (24 h reaction of the silver nitrate solution with the O. *sanctum* leaf extract) on carbon-coated copper TEM grids. The films on the TEM grid were allowed to stand for 2 min, following which extra solution was removed using a blotting paper and grid allowed to dry prior to measurement. TEM measurements were performed on a Phillips EM-CM-12 model instrument operated at an accelerating voltage of 100 KV.

Potentiometric study

The change in the oxidation - reduction potential of the nanoparticles containing solution with time was studied using a potentiometer (Digital potentiometer, 318, Systronics, India) in which saturated calomel electrode and platinum electrode were used.

Zeta potential measurement

The zeta potential of the synthesized nanoparticles was determined

(a) Control (Ag NO_3 Solution) (b) 0 h ($AgNO_3$+ LE) (c) 24th h ($AgNO_3$+ LE)

Figure 1. The leaf extract of *O. sanctum* on colour changes in silver nitrate at different time interval.

Table 1. Effect of leaf extract of *O. sanctum* on colour changes in silver nitrate solution at different time interval.

Time interval	Colour change
0 min	Dark green
10 min	Pale green
30 min	Reddish green
1h	Red
2 h	Red
4 h	Reddish brown
8 h	Reddish brown
16 h	Brown Threads
24 h	Brown Threads

by means of the zeta potential analyzer at pH around 5.5 by suspending the nanoparticles containing solution in potassium chloride solution with ionic strength 10^{-3} M. The measurement of zeta potential is based on the direction and velocity of particles under influence known electric field.

Antimicrobial assay for silver nanoparticles

The antimicrobial assay was performed by the disc - diffusion technique. In this technique, 50 µl of silver nanoparticle prepared from leaf extract, applied to sterile paper discs of 5 mm diameter. The discs were then placed on Nutrient Agar inoculated with clinical strains of bacteria (*Klebsiella pneumoniae*). The plates were incubited at 37^{0}C for overnight. The zone of inhibition was measured in millimeter after the 24 h of incubation and recorded.

Silver nanoparticles loading on cotton fabrics

Cotton fabrics were washed, sterilized and dried before use. Experiments were performed on samples with maximum dimensions of 5 × 5 cm. In order to impregnate cotton fabrics (5 × 5 cm), these were submersed in an Erlenmeyer (50 ml) flask containing silver nanoparticles solution and shaking at 600 rpm for 24 h and dried at 70°C.

Antibacterial activity of nanoparticles loaded on cotton fabrics

To examine the bacterial growth or death kinetics in the presence of silver nanoparticles loaded fabric, *E. coli* cells were grown in continuously stirred 100 ml LB medium at 37°C supplemented by a pre-weighed piece of fabric and agitated at 200 RPM. Growth kinetics rates and bacterial concentrations were determined by measuring the optical density (OD) at 600 nm. The OD values were converted into concentrations of *E. coli* colony forming units (CFU) per ml) using the approximation that an OD value of 0.1 corresponded to a concentration of 108 cells per ml (Pal et al., 2007).

RESULTS AND DISCUSSION

The color change was noted by virtual observation in *O. sanctum* leaf extract incubated with an aqueous solution of $AgNO_3$. It started to change color from watery to yellowish brown at 4th h and dark pink color at the 24th h after incubation (Figure 1). It is due to the reduction of silver ions, this exhibit the formation of silver nanoparticles (Table 1). The color of the extract changed to intense brown after 24 h of incubation and there was no significant change afterwards (Figure 1).

The presence of silver nanoparticles was confirmed by obtaining a range of 200 to 600 nm. A typical peak of λ_{max} at 421 nm was obtained due to the surface plasmon resonance of silver nanoparticles. The surface plasmon absorption peaks depends on the size and shape of the metal nanoparticles as well as on the dielectric constant of the metal itself and the surrounding medium (Mukherjee et al., 2002) (Figure 2).

FTIR measurements were carried out to identify the possible bio-molecules responsible for capping and efficient stabilization of the metal nanoparticles synthesized by leaf extract. The silver nanoparticle sample shows peaks at3313.48,3193,2976.90,2883,1670,1452,1338,1196.78, and 1112.75 cm^{-1} (Figure 3).

The peaks corresponding to protein and silver nanoparticles were found commonly present in the nanoparticles synthesized by leaf extract. The peaks observed for silver nanoparticles at 1678 cm^{-1} (C=C groups or from aromatic

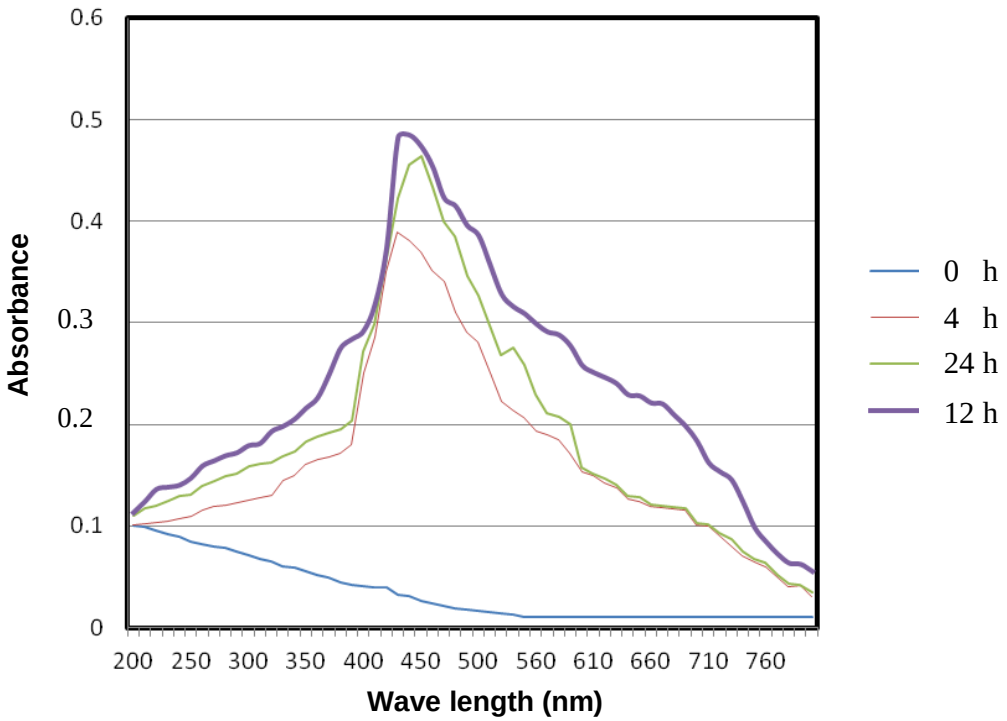

Figure 2. UV-Visible spectra of silver nanoparticles synthesized through leaf extract of *O. sanctum* at different time interval.

rings), 1338 cm^{-1} (germinal methyls), and 1112 cm^{-1} (ether linkages), 3400 to 3200 cm^{-1} and 3000 to 2850 cm^{-1} were assigned to O-H stretching of alcohol and phenol compounds and aldehydic -C-H- stretching of alkenes, respectively. The peaks in the region of 1640 to 1550 cm^{-1} and 1450 to 1375 cm^{-1} correspond to N-H (bend) of primary and secondary amides and C-H (-CH3 - bend) of alkanes, respectively (Kannan and Subbalaxmi, 2011; Sathyavathi et al., 2010).

The peaks in the region of 1350 to 1000 cm^{-1} correspond to -C-N- stretching vibration of the amine or -C-O- stretching of alcohols, ethers, carboxylic acids, esters and anhydrides. FT-IR analysis reveals that the carbonyl group from amino acid residues and proteins has the strong ability to bend metal indicating that the proteins could possibly form a layer covering the metal nanoparticles (that is, capping of silver nanoparticles) to prevent agglomeration and thereby stabilize the medium (Khabat Vahabi et al., 2011; Udayasooriyan et al, 2011).

The crystalline nature of silver nanoparticles was studied with the aid of X-ray diffraction as shown in Figure 4. A number of strong Bragg's diffracted peaks observed at 27.82, 32.25, 46.22 and 76.63 corresponding to the 126, 199, 131 and 24 height of the face centered cubic pattern of silver were obtained. It suggests that the synthesized silver nanoparticles are crystalline in nature. The size of the silver nanoparticles was found to be 26 nm; and it was through by using the width of the (126) Bragg's reflection. In addition, yet some unassigned peaks were also observed suggesting the crystallization of biophase occurs on the surface of silver nanoparticles. X-ray diffraction (XRD) pattern thus clearly shows that the silver nanoparticles formed from phyto-mediated synthesis is crystalline in nature (Harekrishna et al., 2009).

Figure 5 shows fluorescence emission spectrum from silver nanoparticles, dispersed in double distilled water. A strong maximum at 431 nm wavelength and a quantum yield was 666.450 mV appeared in the fluorescence emission spectrum of *O. sanctum* leaf extract mediated silver nanoparticles. The pattern of the emission spectrum revealed that the visible emission from a silver particle is due to a transition of a photo generation electron from the conduction band to a deeply trapped hole (Liu et al., 2004).

The reduction of silver ions to form nanoparticles was also monitored using a potentiometer. A sharp reduction in the potential could be observed upon 4 h of interaction further indicating the formation of nanoparticles at this stage. The potential down from an initial value of 0.436 V for silver ions to 0.153 V at the end of 11 h (Figure 6) after which the fall in potential was gradual, falling up to 0.048 V at the end of 24 h. The result obtained further corroborated the result from UV-visible spectroscopic studies that the Ag reduced at 11 h. Silver nanoparticle solutions will always have a residual charge unless the redox potential of the reductant used is

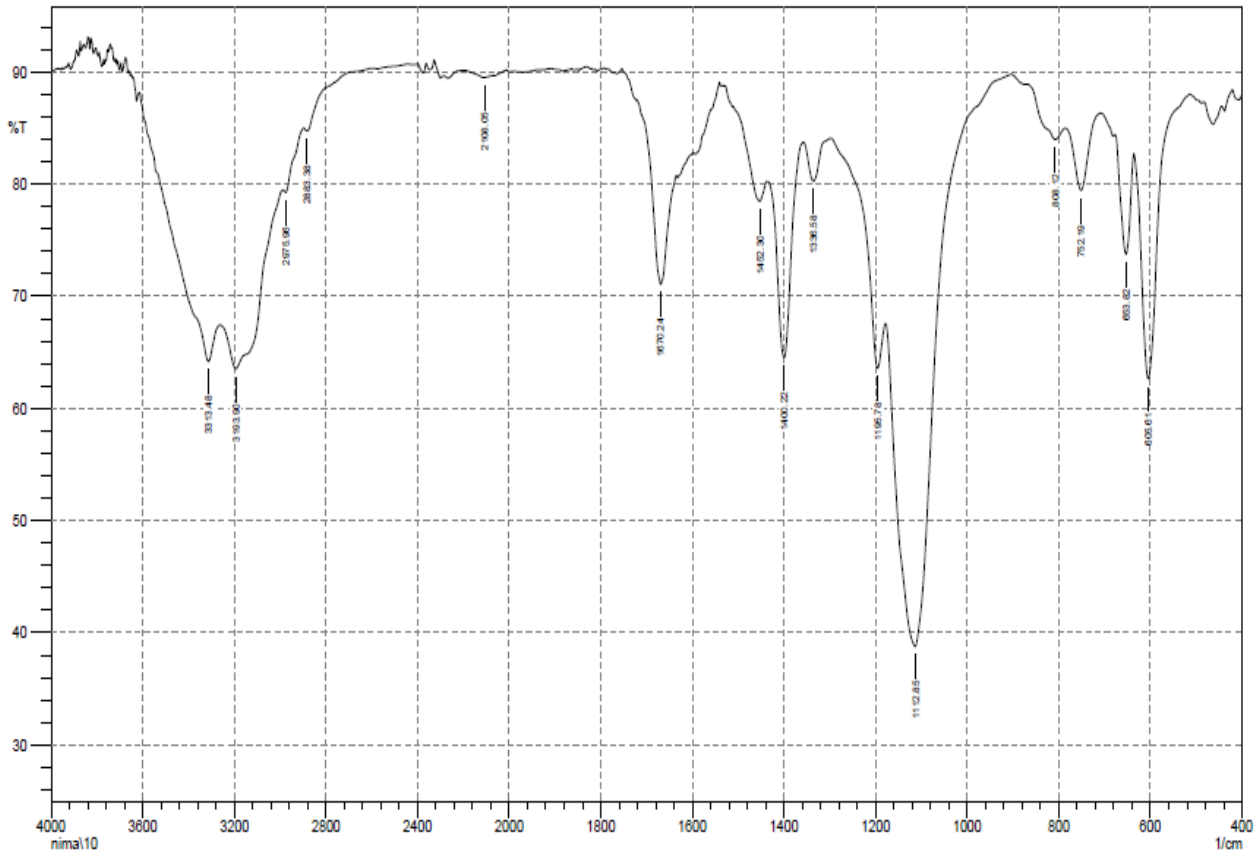

Figure 3. FTIR spectra of silver nanoparticles synthesized from the leaf extract of *O. sanctum*.

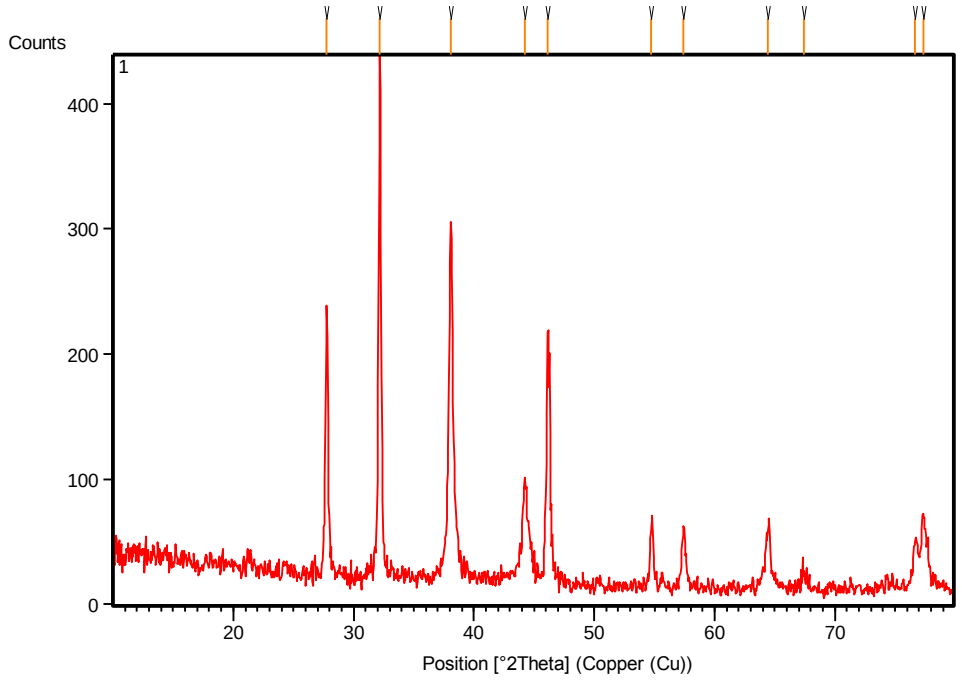

Figure 4. X ray Diffraction analysis on the silver nanoparticles synthesized from the leaf extract of *O. sanctum.*

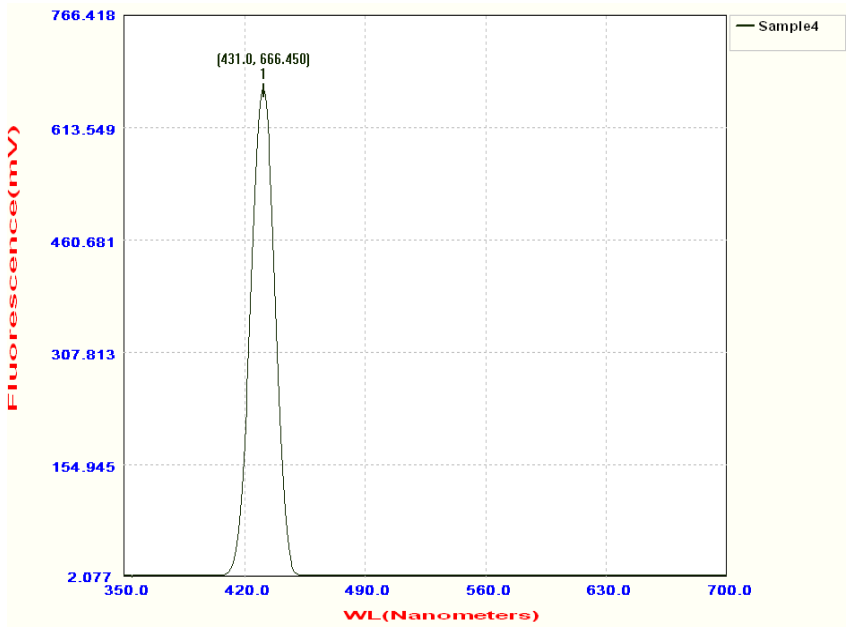

Figure 5. Specrofluorimetric analysis of silver nanoparticles synthesized from the leaf extract of *O. sanctum*.

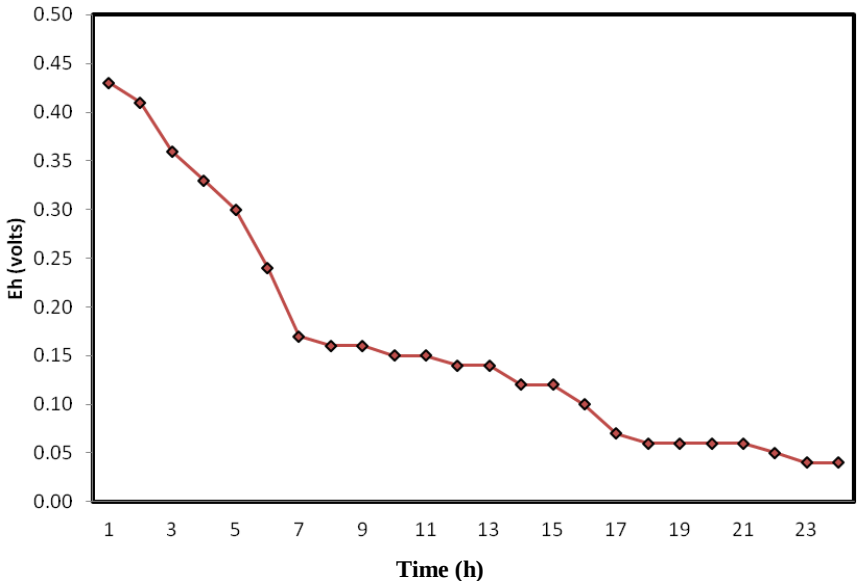

Figure 6. Potentiometric analysis of nanoparticles at functional time for synthesis of nanoparticles from leaf extracts *O. sanctum*.

identical to -0.7 NHE (Mulvaney, 1996).

The alteration in zeta potential with a moment in time is shown in Figure 7. It can be observed that there was a charge stabilization from 11 to 16 h, with the charge stabilized around -57 mV. The zeta potential was -62 mV for the 14 h interacted samples which further decreased to -35 mV for the 24 h interacted samples. Solutions with zeta potential above +25 mV or below -25 mV are considered stable (Su et al., 2010). The stability is determined by the surface charge density and an increase in surface charge density decreases the tendency of aggregation and vice versa (Gast, 1977). Minor changes in the measured zeta potential sometimes may indicate significant changes in the surface charge density (El Badawyet al., 2010).

The results obtained from visible color changes, UV-

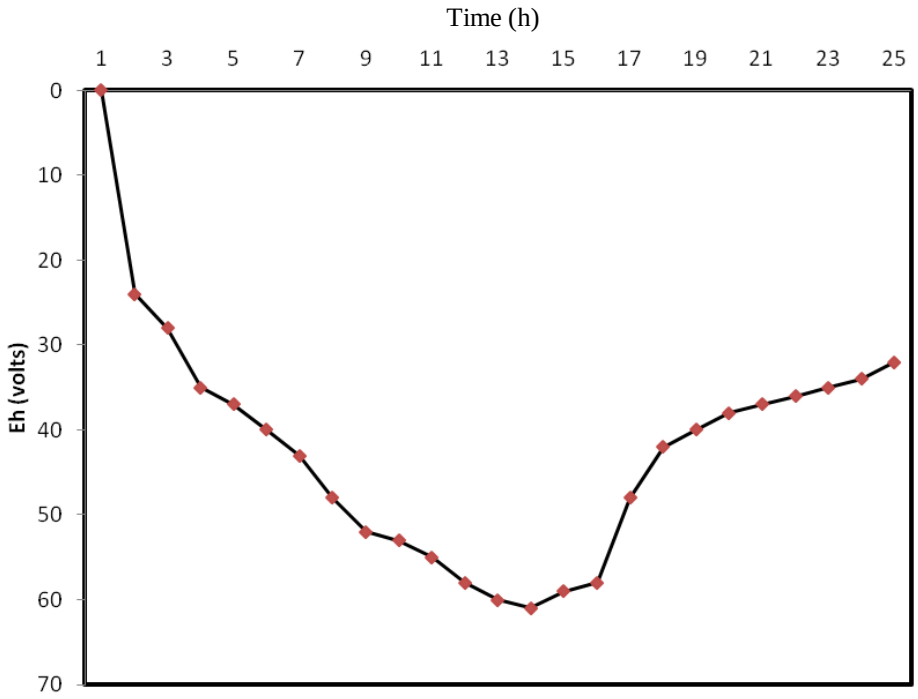

Figure 7. Zeta potential analysis in the formation of silver nanoparticles by leaf extract of *O. sanctum.*

visible studies, potentiometry, and zeta potential studies typically prove that silver nanoparticles started to appear in the system at the end of 4 h. Another significant conclusion drawn was that there was a notable agglomeration in the system at 12 h of interaction as observed from XRD and zeta potential analysis.

The shape and size of silver nanoparticles were analyzed after 24 h of incubation using SEM is shown in Figure 8. In general, the nanoparticles were in spherical shape with varying size ranged from 7 to 28 nm. Most of the nanoparticles were combined with only a few of them were scattered, as observed under SEM.

The energy dispersive spectrum (Figure 9) revealed the clear identification of the elemental composition profile of the synthesized nanoparticles, which suggests the presence of silver as the ingredient element. The EDS spectrum showed high for silver signals. The vertical axis shows the counts of the X-ray and the horizontal axis shows energy in keV. The strong signals of silver correspond to the peaks in the graph confirming presence of silver.

TEM technique was employed to visualize the size and shape of Ag nanoparticles. Figure 10 shows the typical TEM micrograph of the synthesized Ag nanoparticles. It is observed that most of the Ag nanoparticles were spherical in shape. A few agglomerated silver nanoparticles were also observed in some places, thereby indicating possible sedimentation at a later time. It is evident that there is variation in particle sizes and the average size estimated was 26 nm and the particle size ranged from 8 to 45 nm. The natural products, namely glycosides, flavanones, and reducing sugars are the main constituents of the O. sanctum leaf extract. The aldehydic groups and reducing sugars are responsible to the reduction of Ag+ ions into metallic Ag0 and also stabilizing the resulting nanoparticles (Zaheer Khan et al., 2012). The results of experiments may conclude that the *O. sanctum* leaf extract acts as reducing, stabilizing, and capping agents.

Antimicrobial activities of silver nanoparticles synthesized by leaf extracts from *O sanctum* are analyzed. The antibacterial activity of silver nanoparticles showed, the inhibition zone of 12 mm diameter was formed against *K. pneumoniae* by Ag nanoparticles synthesized by leaf extract (Figure 11). The fiber surfaces of the finished fabrics were observed by SEM. Images of the samples in Figure 12 show the deposi-tion of nanoscaled silver particles on the textile surface. The particles size varies from 12 m to 28 nm as can be seen.

The growth and death kinetics of Ag nanoparticles loaded cotton fabrics against E. coli was depicted in Figure 13. The antibacterial action of plain fabric (control), showed a dense population of bacterial colonies. The Ag-loaded fabric showed antibacterial activity it inhibits the growth of bacteria. The results of these antibacterial tests (Figure 13), clearly indicated that the fabric prepared by immersing in Ag nano-particles demon-

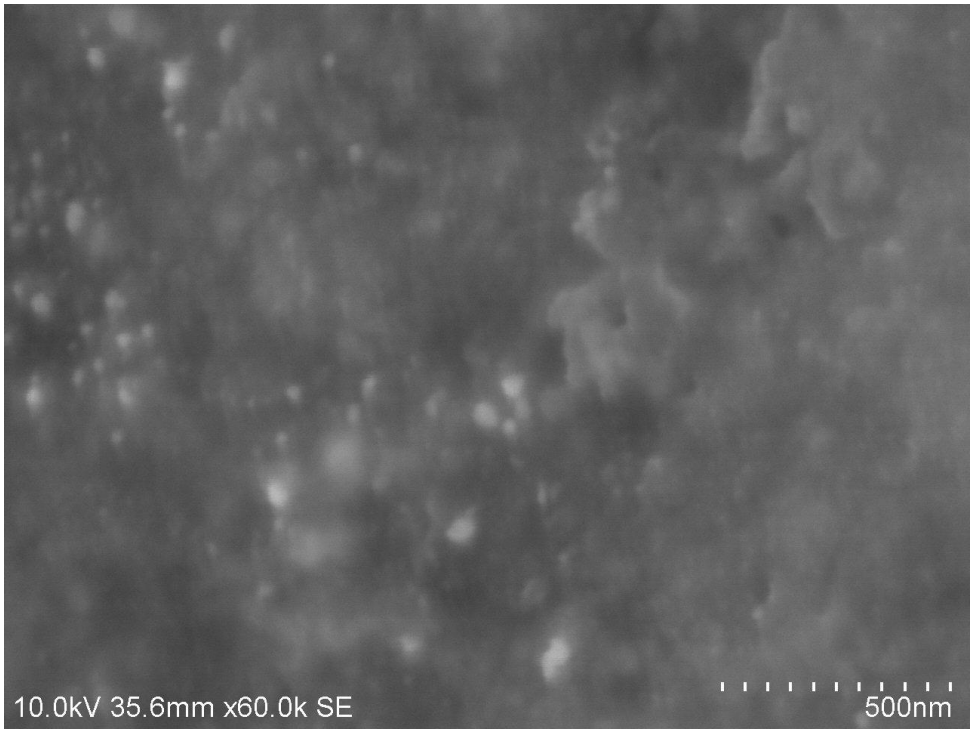

Figure 8. SEM images of silver nanoparticles by leaf extract of *O. sanctum.*

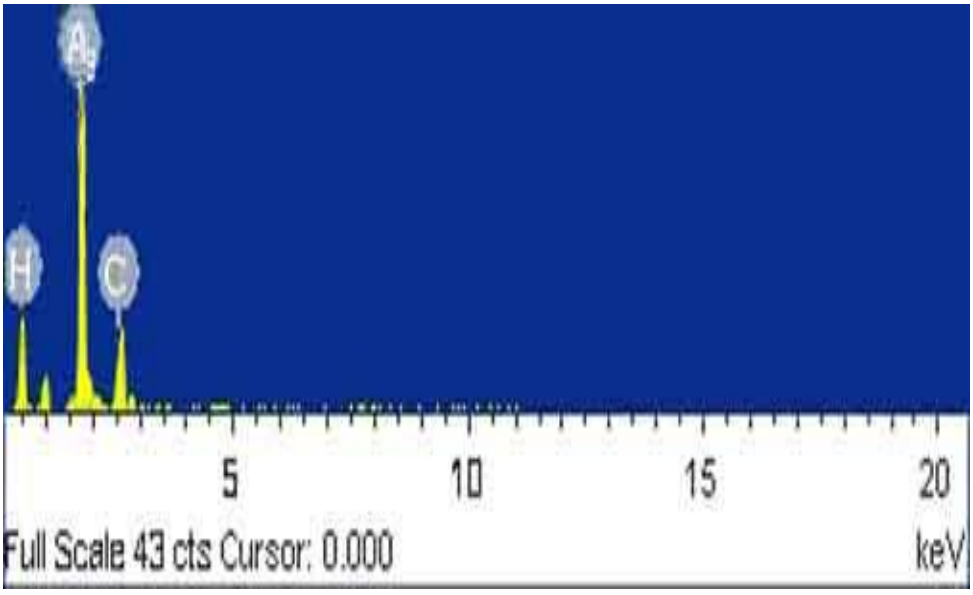

Figure 9. EDAX spectra of silver nanoparticles synthesized from leaf extract of *O. sanctum.*

demonstrated greater biocidal activity. Therefore, the Ag content of the fabric is a key factor in controlling its antibacterial activity. It is clear from Figure 13 that in the initial phase bacterial growth is almost the same in the media containing plain and Ag-loaded fabrics. The killing action of Ag-loaded fabric began approximately 4 h after its incubation in nutrient broth medium. This may be attributed to the fact that when the Ag-loaded fabric was put in the bacterial suspension, the fabric began to expose to bacteria and killed the bacterial cells (Pranee et al., 2008). The killing activity of Ag-loaded fabric began to be reduced later, perhaps because nearly

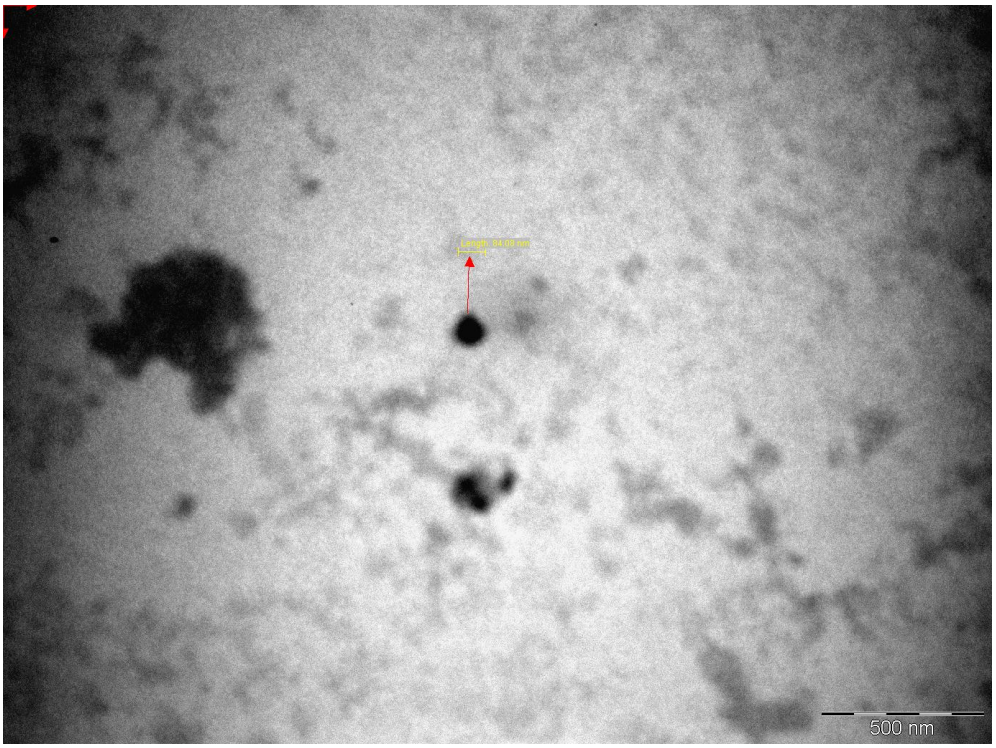

Figure 10. TEM image of silver nanoparticles by leaf extract of *O.sanctum.*

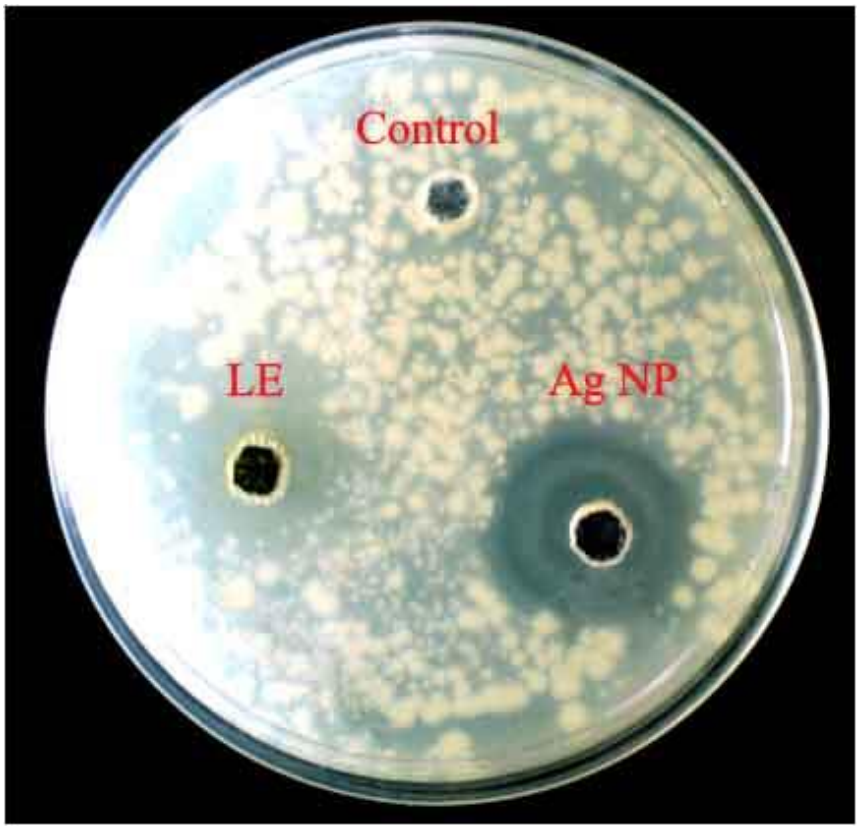

Figure 11. The antibacterial effect of silver nanoparticles synthesized from the leaf extract of *O. sanctum*.

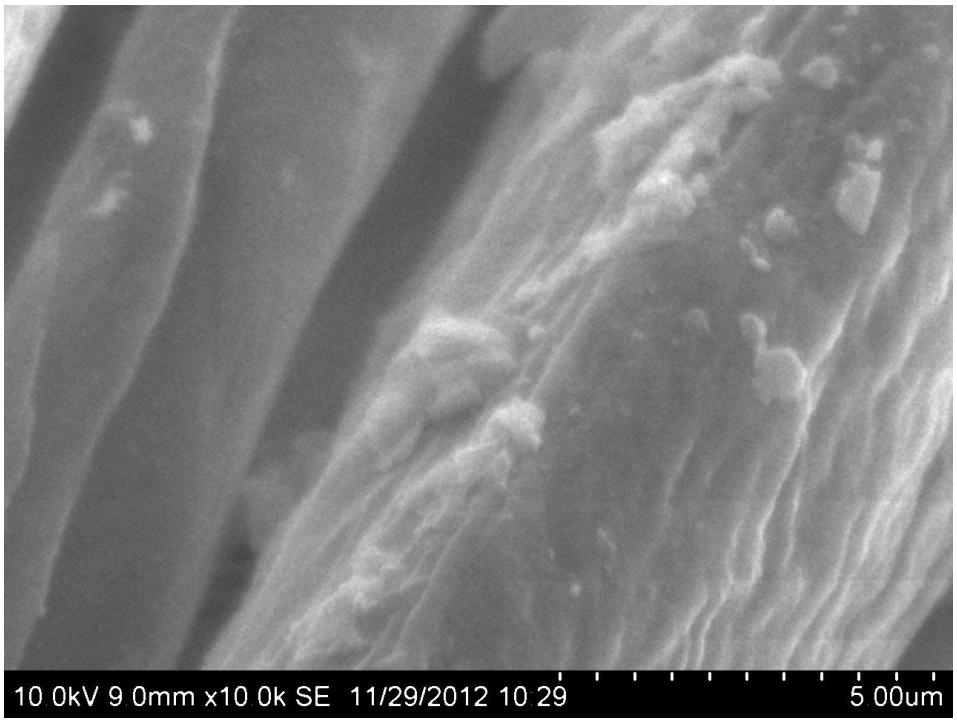

Figure 12. SEM image of nano silver coated Cotton fabric.

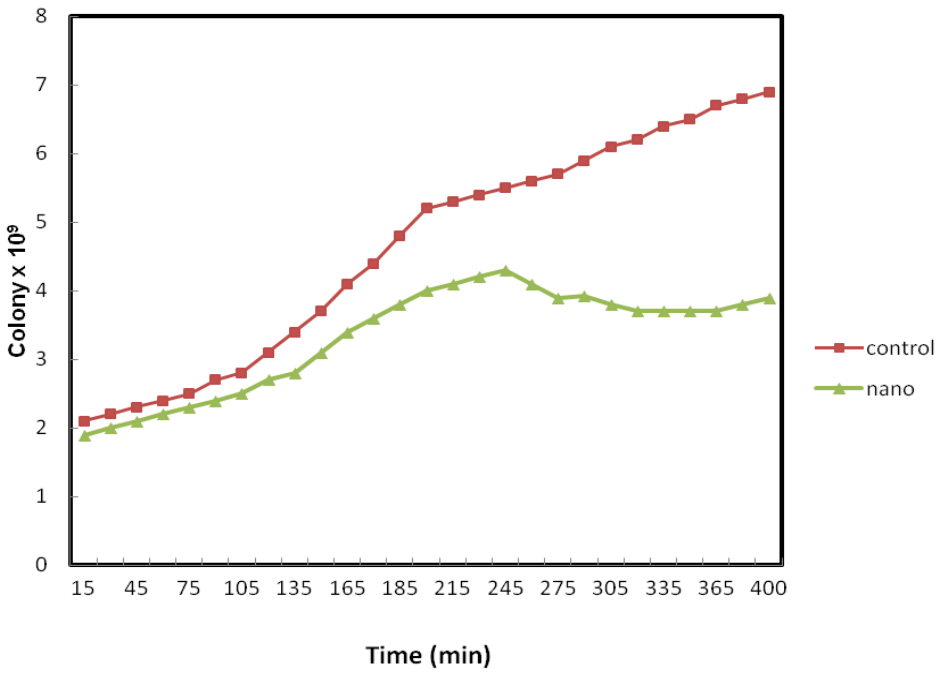

Figure 13. Growth kinetics of Bacterial culture in LB medium in the presence of nanoparticles coated cotton fabrics and control.

all the Ag nanoparticles were consumed in binding to bacterial cells. These findings proved with the experimental data reported by Lee et al. (2003) and Yeo et al. (2003). Kim et al. (2007) and Pranee et al. (2008) suggested that the inhibitory activity of silver nanoparticles was influenced by free radical generated on the

surface of silver nanoparticles.

Conclusion

Silver nanoparticles have been synthesized from the *O. sanctum* leaf extract. Structural analysis by XRD together with the chemical composition by EDS, strongly suggests the formation of elemental silver nanoparticles instead of their oxides. From the TEM analysis, the sizes of the nanoparticles are found to be 5 to 60 nm. FTIR measurements provided strong evidence for proteins to form a coat covering the silver nanoparticles to stabilize and prevent the agglomeration of the particles. This simple procedure for the biosynthesis of silver nanoparticles has several advantages such as cost-effectiveness, compatibility and eco-friendliness for biomedical and pharmaceutical applications.

The phyto mediated silver nanoparticles and Ag nanoparticles treated fabrics show durable significant antimicrobial activity against two common infectious bacteria, namely *K. pneumoniae* and *E. coli*. The synthesis of phyto-mediated silver nanoparticles from *O. sanctum* is useful for application of dressing materials, delicate fabrics, knitted materials etc.,

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Full Length Research Paper

Inhibition of *Salmonella* Typhimurium by bovicin HC5 associated with chelating agents and surfactants

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The antimicrobial effect of bovicin HC5, a bacteriocin produced by *Streptococcus bovis* HC5, associated with chelating agents or surfactants was investigated against *Salmonella* Typhimurium. The microorganism grown in brain and heart infusion broth (BHI) was treated with bovicin HC5 at concentrations of 50, 100, 150 and up to 200 AU.mL^{-1}; associated with chelating agents, or surfactants and growth was assessed by absorbance at 630 nm. Bovicin HC5 did not affect the growth of the microorganism, but when combined with chelating agents or surfactants there was inhibition or reduction of growth. The association of bovicin HC5 with EDTA promoted the highest growth inhibition and bactericidal effect after 2.5 h. Gluconic acid, nitrile-tri-acetic acid, diethanolamine, sodium citrate, lactic acid, ramnolipid and Tween 80 reduced the specific growth rate of *Salmonella,* in at least one of the concentrations of the bovicin HC5 tested. Therefore, the bactericidal effect of bovicin HC5 against *Salmonella* Typhimurium to is augment in the presence of chelating agents and surfactants, which possibly act in the destabilization of the bacterial outer membrane, which allows the action of the bacteriocin on cell membranes.

Key words: Bovicin HC5, bacteriocin, *Salmonella* Typhimurium.

INTRODUCTION

Bioconservation explores the use of microorganisms and/or their metabolites to increase shelf life and ensure food safety, with minimum impact on the nutritional properties and sensory characteristics of products (García et al., 2010; Gálvez et al., 2010). In this context, the use of bacteriocins stands out because these are considered as a components of the foods (Allende et al., 2007; Settanni and Corsetti, 2008), and they are present naturally in products fermented by lactic bacteria (Allende et al., 2007). Evidence of their degradation by gastro-intestinal proteases indicates safety to consumers' health (Holzapfel et al., 1995; Allende et al., 2007). Several have been evaluated as important strategy for the control of pathogenic and spoilage microorganisms in foods (Allende et al., 2007).

Bacteriocins are peptides synthesized ribossomically (Meghrous et al., 1999; Héchard and Sahl 2002), by Gram-negative and Gram-positive bacteria, released into the extracellular medium where they display bacteriostatic or bactericidal effects on other bacteria (Meghrous et al., 1999; Arqués et al., 2011). They belong to a heterogeneous group of peptides with variations in the spectrum of activity, biochemical properties, molecular weight, mode of action, and genetic source (Abee et al., 1995).

Bovicin HC5 is a bacteriocin from the group of lantibiotics produced by *Streptococcus bovis* HC5, a bacterium isolated from the rumen of cattle (Mantovani and Russel, 2003). Bovicin HC5 inhibits Gram-positive bacteria such as *Listeria monocytogenes* (Mantovani and Russel, 2003), *Bacillus cereus*, *Bacillus thuringiensis*

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(Carvalho et al., 2007a), *Clotridium tyrobutyricum* (Carvalho et al., 2007b) and *Alicyclobacillus acidoterrestris* (Carvalho et al., 2008).

However, Gram-negative bacteria such as *Salmonella* are generally resistant to the action of bacteriocins produced by Gram-positive bacteria (Cao-Hoang et al., 2008; Arques et al., 2011). Their outer membrane acts as a barrier preventing the diffusion of macromolecules as bacteriocins, enzymes and hydrophobic substances such as antibiotics, and represents a physiologic advantage of this group of bacteria (Alakomi et al., 2000).

The use of chemical compounds, such as chelators or mild heat treatment and freezing may destabilize the outer membrane and thus reduce the resistance of Gram-negative bacteria to the action of bacteriocin (Boziaris and Adams; 1999; Molinos et al., 2008a). This awareness can be exploited to increase the spectrum of action of bacteriocins produced by Gram-positive bacteria, which is important given the high incidence of foodborne diseases caused by Gram-negative pathogens (Gálvez et al., 2010; Burgos et al., 2012). The efficiency of this strategy has already been shown by Molinos et al. (2008a) who observed that the combination of enterocin AS-48 with chemical compounds such as lactic acid, polyphosphoric and peracetic, sodium hypochlorite, chloride hexa-decylpyridinium, and hydrocinnamic acid in washing solutions significantly reduced the counts of *Salmonella* in soybean sprouts by at least 48 h of storage.

This study explored this potential of association of chelating agents and surfactants with bacteriocins to enhance the inhibition of growth of *Salmonella* Typhimurium by bovicin HC5.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Salmonella enterica sorovar Typhimurium ATCC 14028 was grown in brain and heart infusion broth (BHI, Difco) at 37 ± 1°C for 18-20 h. The cells were centrifuged at 1742 *g* (Sorvall RT 6000D) and washed with 0.1% peptone water. *S. bovis* HC5 was cultivated as described by Mantovani and Russel (2003). *L. lactis* ATCC 19435 was cultivated in *Lactobacillus* broth (MRS, Himedia) at 37 ± 1°C and used to determine the antimicrobial activity of bacteriocin.

Preparation and activity of bovicin HC5

Extracts of bovicin HC5 were prepared as described by Mantovani and Russell (2003). The antimicrobial activity was determined with indicator *L. lactis* by the diffusion method in agar (Tagg et al., 1976), quantified by the critical dilution method (Hoover and Harlander, 1993) and expressed in arbitrary units ($AU.mL^{-1}$).

Preparation of solutions of chelating agents and surfactants

The following chemical compounds were used as chelators: ethylenediamine tetraacetic acid (EDTA) (Reagen), sodium citrate (Ecibra), lactic acid (Vetec) nitrile-tri-acetic acid (NTA) (Sigma), gluconic acid (Sigma), diethanolamine (DTA) (Carlos Erba), calcium acetate (Vetec) and dibasic potassium phosphate (Vetec). The solutions were sterilized by filtration through membranes with pores sized 0.2 µm (Millipore ®), except that the EDTA solution was sterilized by autoclaving at 121°C for 15 min. The surfactants used were Tween 80 (Sigma) and ramnolipid (Jeneil). The critical micelle concentration (CMC) of ramnolipid was obtained by construction of a voltage curve in tensiometer Fischer Scientific and sterilized by filtration. The Tween 80 solution was prepared in accordance with data of CMC obtained from the literature (Zhang et al., 2003) and sterilized by autoclaving (121° C, 15 min).

Determination of minimum inhibitory concentration (MIC)

The inhibitory effect of each chelating agent or surfactant was determined by minimum inhibitory concentration (MIC) tests by microdilution technique. To prepare the inoculum, the culture of *S.* Typhimurium was streaked on plate count agar (PCA, Difco), incubated at 37 ± 1°C for 16 to 18 h and colonies were suspended in 0.85% saline solution to achieve a density equivalent to standard solution of McFarland 0.5 (CLSI, 2003). The culture was inoculated at the concentration of 10^5 $CFU.mL^{-1}$ in Mueller-Hinton broth added by the chelating agent or tested surfactant. The following concentrations of each agent were evaluated: EDTA (from 1.6 to 100 $mM.L^{-1}$), gluconic acid and sodium citrate (12.5 to 800 $mM.L^{-1}$), NTA, DTA, calcium acetate and potassium phosphate (6.25 to 400 $mM.L^{-1}$), lactic acid (7.0 to 450 $mM.L^{-1}$) and surfactants Tween 80 and ramnolipid (0.5 to 5.0 times CMC). Aliquots of 125 µL of Mueller-Hinton broth were distributed in 96 wells of microplates, followed by incubation at 37 ± 1°C. The growth was monitored by absorbance at 630 nm at microplate reader (Thermo Plate, model TP – Reader) at different time intervals for a period of 16 to 20 h.

Effect of bovicin HC5 associated with chelating agents or surfactants

The effect of the antimicrobial action of bovicin HC5 associated with chelating agents, or surfactants, was evaluated on microplates. *S.* Typhimurium was activated in BHI broth for 18 to 20 h, washed in 0.1% peptone water, resuspended in 5 mL and inoculated at a concentration of 10^7 $CFU.mL^{-1}$ in BHI broth with the chelating agent or surfactant and bovicin HC5. The concentrations of chelating agents or surfactants used were at least twice smaller than that of the MIC or when this could not be established, a concentration was selected of the agent that did not reduce efficiently the growth rate of the microorganism. The concentrations of bovicin HC5 50, 100, 150 and 200 $AU.mL^{-1}$ were evaluated. The microplates were incubated at 37 ± 1°C and the growth evaluated by absorbance at 630 nm (Thermo Plate, model TP-Reader), at different time intervals, for a period of 24 h.

The specific growth rate (µ) was determined by means of the inclination of the linearized line data line of at least four points in the exponential phase of growth.

Evaluation of the bactericidal effect of bovicin HC5 associated with EDTA on *Salmonella* Typhimurium

To evaluate the bactericidal effect of bovicin HC5 associated with EDTA on *S.* Typhimurium, culture was activated in BHI broth (18 to 20 h, 37 ± 1°C), washed in 0.1% peptone water and suspended in BHI broth with EDTA (1.6 $mM.L^{-1}$) and bovicin HC5 (50 and 200 $AU.mL^{-1}$), at a density of 10^5 $CFU.mL^{-1}$. At different time intervals, aliquots were collected, and plated on PCA, initially by the drop method and as of 180 min, by the spread plate technique of 0.1 mL

Table 1. Susceptibility of *Salmonella* Typhimurium in Mueller-Hinton broth a treatment with chelating and surfactants agents.

Antimicrobial agents	MIC (mM.L^{-1} or x CMC)
Chelants	
Acetate calcium	> 400.0
Acid gluconic	> 800.0
Acid latic	112.5
Nitrile-tri-acetic acid	100.0
Diethanolamine	200.0
Potassium phosphate	> 400.0
Sodium citrate	800.0
EDTA[1]	12.0
Surfactants	
Ramnolipid	> 5 x
Tween 80	> 5 x

EDTA[1] - ethylenediamine tetra-acetic acid.

on surface, in treatments containing bovicin HC5 and EDTA. The Petri dishes containing drops were incubated at 37 ± 1°C for 6-8 h. The inoculated plates by spread plate method were incubated at 37 ± 1°C for 24 h.

Analysis of data

Each treatment was performed twice with a minimum of three replicates. Statistical analyses were performed in SAEG program, version 9.1, 2007 (Federal University of Viçosa, 2007). Variance analysis and Tukey's or Dunnett's tests were used to check the existence of differences between treatments and the control group. The level of statistical significance adopted was 0.05.

RESULTS

MIC of chelating agents and surfactants

The growth of *S.* Typhimurium in Mueller-Hinton broth was used to compare the inhibitory effect of different concentrations of chelating agents used. EDTA presented higher antimicrobial effect and inhibited bacterial growth at lower concentrations than the other agents used and MIC was 12 mM.L^{-1} (Table 1). Nitrilo-tri-acetic acid and lactic acid suppressed growth at concentrations of 100 and 112.5 mM.L^{-1}, respectively (Table 1). The MIC's of diethanolamine (DTA) was 200 mM.L^{-1} and at concentration of 100 mM.L^{-1}, there was a reduction of growth rate (Table 1).

Sodium citrate inhibited the growth of *S.* Typhimurium at the concentration of 800 mM.L^{-1} and reduced the growth rate at concentrations of 200 and 400 mM.L^{-1}, whereas calcium acetate only reduced the growth rate in these concentrations (Table 1).

Gluconic acid and potassium phosphate had no significant effect on the growth of this microorganism, at the concentrations evaluated, thus it was not possible to determine the MIC (Table 1).

The ramnolipid surfactant and Tween 80 used in this study did not inhibit the growth of *S.* Typhimurium, even at concentrations above the critical micelle concentration (CMC), that is, when the surfactant is already capable of forming micelles (Table 1).

These results allowed the selection of concentrations of chelating agents and surfactants that would not exert influence on the growth of *S.* Typhimurium to be then, evaluated in combination with bovicin HC5. The concentrations chosen were at least two times smaller than MIC, when it could be determined.

Effect of bovicin HC5 associated with chelating agents or surfactants

Separately, concentrations of up to 200 AU.mL^{-1} bovicin HC5 in BHI broth did not affect significantly ($p>0.05$) the growth of *S.* Typhimurium. However, when combined with chelating agents, there was inhibition or reduction in the specific growth rate (Table 2). A synergic effect of bovicin HC5 was noted with diethanolamine, nitrile-tri-acetic acid and gluconic acid, whose association caused a reduction in the growth rate of *S.* Typhimurium, independent of the concentration of bacteriocin used (Table 2). When associated to potassium phosphate and calcium acetate, bovicin HC5 did not inhibit bacterial growth even at concentrations of 200 AU.mL^{-1} (Table 2). In association with lactic acid, bovicin HC5 reduced bacterial growth at the largest concentrations used, 150 and 200 AU.mL^{-1} (Table 2).

Bovicin HC5 at the concentration of 200 AU.mL^{-1} did not affect the growth of *S.* Typhimurium (Figure 1), indicating the resistance of the microorganism to the action of the bacteriocin, possibly by the presence of the outer membrane. However, in the presence of 1.6 mM.L^{-1} of EDTA, the lowest concentration of bovicin HC5 (50 AU.mL^{-1}) completely inhibited the growth of *S.* Typhimurium during 48 h of incubation (Figure 1).

The surfactants used in combination with bovicin HC5 slowed growth of *S.* Typhimurium, independent of the concentration of bacteriocins used (Table 3).

These results indicate that the effect of bovicin HC5 on the growth of *S.* Typhimurium varied depending on the agent used in combination, and show that EDTA to be promising in exerting exert a synergistic effect with bovicin HC5.

Bactericidal effect of bovicin HC5 associated with EDTA on *S.* Typhimurium

Although initially bovicin HC5 associated with EDTA has inhibited the growth of *S.* Typhimurium, after 150 min of treatment, it was possible to confirm the bactericidal effect from the reduction of viable cell count (Figure 2). This effect was more accentuated at the highest concentration of bovicin HC5 used (200 AU.mL^{-1}). At this concentration, the counts were below the limit of detection

Table 2. Specific growth rate μ (h^{-1}) of *Salmonella* Typhimurium in BHI broth exposed to different concentrations of bovicin HC5 associated with chelating agents incubated at 37 ± 1°C for 48 h.

Chelating agent	μ (h^{-1})Concentration of bovicin HC5 (AU.mL^{-1})				
	0	50	100	150	200
Etylenodiamine tetraacetic acid (1.6 mM.L^{-1})	$^{a}0.52^{C}$	$^{b}0^{C}$	$^{b}0^{C}$	$^{b}0^{C}$	$^{b}0^{C}$
Nitrile-tri-acetic acid (6.25 mM.L^{-1})	$^{a}0.78^{AB}$	$^{b}0.55^{AB}$	$^{b}0.49^{AB}$	$^{b}0.46^{A}$	$^{b}0.45^{AB}$
Lactic acid (7.0 mM.L^{-1})	$^{ab}0.77^{AB}$	$^{ab}0.64^{AB}$	$^{ab}0.63^{AB}$	$^{b}0.51^{A}$	$^{b}0.47^{AB}$
Calcium acid (12.5 mM.L^{-1})	$^{a}0.73^{AB}$	$^{a}0.57^{AB}$	$^{a}0.67^{AB}$	$^{a}0.60^{A}$	$^{a}0.55^{AB}$
Diethanolamine (50 mM.L^{-1})	$^{a}0.71^{B}$	$^{b}0.42^{B}$	$^{b}0.38^{AB}$	$^{b}0.47^{A}$	$^{b}0.40^{B}$
Sodium Citrate (100 mM.L^{-1})	$^{a}0.83^{AB}$	$^{b}0.55^{AB}$	$^{c}0.32^{B}$	$^{d}ND^{B}$	$^{d}ND^{C}$
Gluconic Acid (100 mM.L^{-1})	$^{a}0.94^{AB}$	$^{b}0.67^{AB}$	$^{b}0.68^{AB}$	$^{b}0.61^{A}$	$^{b}0.61^{AB}$
Potassium Phosphate (100 mM.L^{-1})	$^{ab}0.84^{AB}$	$^{b}0.63^{AB}$	$^{ab}0.66^{AB}$	$^{ab}0.65^{A}$	$^{ab}0.66^{AB}$

ND, Not determined. $^{A-C}$ Averages followed by the same letter in the column do not differ between themselves by the Tukey's test (p<0,05). $^{a-d}$ Averages followed by the same letter in the line do not differ between themselves by the Tukey's test (p<0.05).

Table 3. Specific growth rate μ (h^{-1}) of *Salmonella* Typhimurium in BHI broth exposed to different concentrations of bovicin HC5 associated with surfactants incubated at 37 ± 1°C for 48 h.

Surfactant	μ (h^{-1}) Concentration of bovicin HC5 (AU.mL^{-1})				
	0	50	100	150	200
Ramnolilip (5x CMC)	0.83^{A}	0.68^{B}	0.68^{B}	0.70^{B}	0.41^{B}
Tween 80 (5x CMC)	1.01^{A}	0.64^{B}	0.66^{B}	0.66^{B}	0.62^{B}

$^{A-B}$ Averages followed by the same letter in the line do not differ between themselves by Dunnett's test (p<0.05).

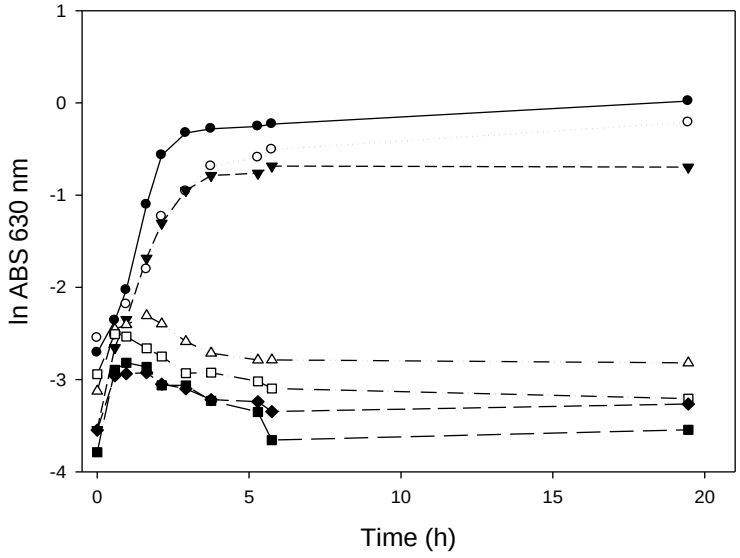

Figure 1. Growth of *S.* Typhimurium in BHI broth exposed to treatment with different concentrations of bovicin HC5 and EDTA, incubated at 37 ± 1 °C for 24 h. BHI broth (—●—); BV HC5 (200 AU.mL^{-1}) (..○..); EDTA (1.6 mM.L^{-1}) (--▼--); EDTA + BV HC5 (50 AU.mL^{-1}) (—..Δ..—); EDTA + BV HC5 (100 AU.mL^{-1}) (—■—); EDTA + BV HC5 (150 AU.mL^{-1}) (—..□..—); EDTA + BV HC5 (200 AU.mL^{-1}) (——♦——).

of the technique after 180 min of treatment (Figure 2).

This same pattern of behavior was observed when cells of *S.* Typhimurium were treated with EDTA and the lowest concentration of bovicin HC5, with a difference of approximately 30 min to the beginning of the reduction of the counts and 60 min so this reduction fell below the detection

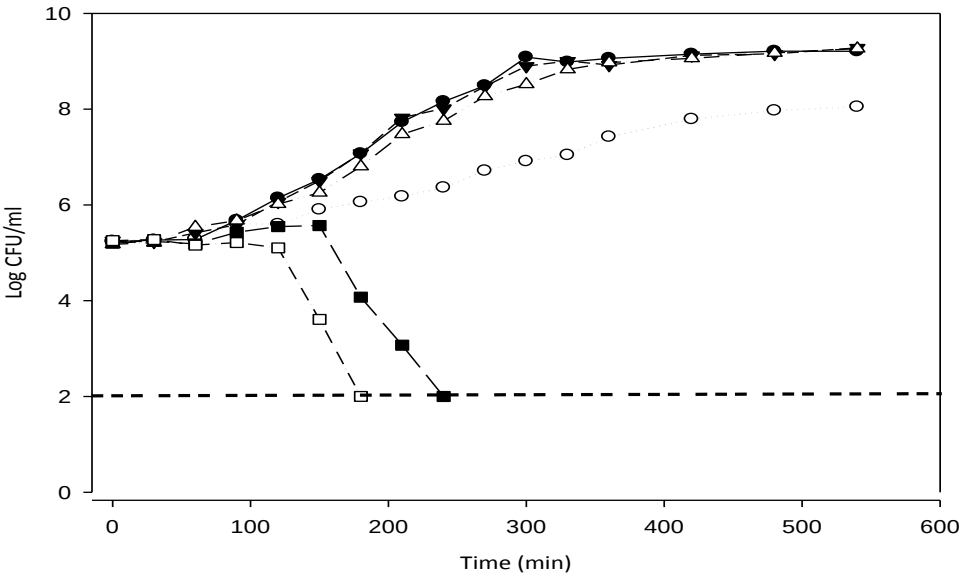

Figure 2. Counts of *Salmonella* Typhimurium treated with EDTA and bovicin HC5 incubated at 37 ± 1 °C for 540 min. *S.* Typhimurium (—●—); *S.* Typhimurium + EDTA (1.6 mM.L^{-1}) (..○..); *S.* Typhimurium + bovicin HC5 (50 AU.mL^{-1}) (--▼--); *S.* Typhimurium + bovicin HC5 (200 AU.mL^{-1}) (—..Δ..—); *S.* Typhimurium + EDTA (1.6 mM.L^{-1}) + bovicin HC5 (50 AU.mL^{-1}) (—■—); *S.* Typhimurium + EDTA (1.6 mM.L^{-1}) + bovicin HC5 (200 AU.mL^{-1}) (—..□..—), detection limit of the technique (**- - -**).

limits of the technique (Figure 2).

As observed in previous experiments, there was no difference ($p>0.05$) between the behavior exhibited by cells of *S.* Typhimurium in the absence or presence of 50 or 200 AU.mL^{-1} bovicin HC5^{-1} (Figure 2). However, the addition of 1.6 mM.L^{-1} of EDTA exerted partial inhibition in growth, with the increase of lag phase and reduction in the final population in a logarithmic cycle ($p<0.05$).

DISCUSSION

S. Typhimurium is a Gram-negative pathogen of great public health interest. Together with *Salmonella* Enteritidis it is related to almost 75% of reported cases of salmonellosis reported (Lavigne and Blanc-Potard, 2008). As well as the other Gram-negative bacteria, it is naturally more resistant to the action of several antimicrobial compounds, particularly bacteriocins of Gram-positive bacteria (Thongbai et al., 2006). The outer membrane of this group of bacteria acts as a barrier that prevents the diffusion of the bacteriocin molecules up to the cytoplasmic membrane where the peptide exerts its action (Thongbai et al., 2006). As demonstrated by Gänzle et al. (1999), wild strains of *Escherichia coli* and *S.* Typhimurium without any change in the layer of lipopolysaccharide were resistant to the action of nisin and curvacin A, whereas mutant strains with alterations in the deepest layer of lipopolysaccharides, that is prior to the galactose residue in the inner polysaccharide, were sensitive to the action of both bacteriocins.

The use of destabilizing agents of membrane allows the action of such antimicrobial peptides in Gram-negative bacteria (Belfiore et al., 2007; Martin-Visscher et al., 2011). The results obtained showed that the combination of bovicin HC5 with agents such as EDTA, lactic acid, nitril-tri-acetic acid and diethanolamine caused reduction in the growth rate of *S.* Typhimurium. The greater efficiency of association of bovicin HC5 with EDTA confirms the results achieved of this chelating agent in the sensitization of Gram-negative bacteria to the action of bacteriocins of Gram-positive bacteria (Branen and Davidson, 2004). Boziaris and Adams (1999) showed the effect of nisin associated to 10 mM.L^{-1} of EDTA in the growth of *E. coli* ATCC 25922 and noted that, even at low concentrations of bacteriocin (100 AU.mL^{-1}), there was a inhibition of growth of the microorganism. Phillips and Duggan (2001) treated cells of *Arcobacter butzleri* (10^6 CFU.mL^{-1}) with a 20 mM.L^{-1} EDTA and 500 AU.mL^{-1} nisin for 30 min and observed that the simultaneous addition of two components was more efficient for the reduction of counts of viable cells both in the exponential phase and in the stationary phase, with a reduction of 5.6 and 5.1 logarithmic cycles, respectively.

Besides bovicin HC5, other bacteriocins show inhibitory effect on *Salmonella* in the presence of EDTA. *S.* Enteritidis was partially sensitive to treatment with 3200 AU.mL^{-1} cerein 8A, a bacteriocin produced by *B. cereus*, and the addition of EDTA (20, 50 or 100 mM.L^{-1}) significantly increased the bactericide effect of this bacteriocin (Lappe et al., 2009). Recently, Martin-Visscher et al. (2011) found that the association of EDTA (40 mM.L^{-1}) with nisin and gallidermin, at concentrations from 6.25 to 50 µM, inhibited the growth of *S.* Typhimurium ATCC 14207 after 1 h of

treatment at 37°C.

Similar results were also obtained in experiments with food. Economou et al. (2009) treated samples of chicken with 500 and 1500 AU.g^{-1} of nisin and 10 and 50 mM.L^{-1} EDTA for 30 min and observed that this treatment significantly reduced the counts of aerobic mesophilic microorganisms, *Pseudomonas* sp. and Enterobacteriaceae, during the 24 days of storage at 4°C.

EDTA is reported to cause a disruption at least partial in the layer of lipopolysaccharide (Alakomi et al., 2003), possibly due to binding to calcium and magnesium ions to establish crossed connections with residues of sugars and phosphate radicals within the cytoplasmic core, which would reinforce the structure (Alakomi et al., 2003; Branen and Davidson, 2004).

Our results indicate that the action of low concen-trations of EDTA (1.6 mM.L^{-1}) on *S.* Typhimurium was effective to allow the action of bovicin HC5 on the micro-organism, which reduced its growth and even kills the bacteria (Figures 1 and 2). Other chemical compounds also enhanced the action of bovicin HC5, because although the growth of *S.* Typhimurium had not been completely inhibited, diethanolamine, gluconic acid, sodium citrate, lactic acid and nitril-tri-acetic acid (NTA) reduced significantly the specific rate of growth of the pathogen in at least one of the tested concentrations (Table 2). The action mechanism of each agent is not yet well understood, but possibly they would be related to disrupting the structure of the outer membrane. In fact, organic acids or salts can also enhance the action of bacteriocins (Gálvez et al., 2007), as shown by the reduction of the counts of *Staphylococcus aureus,* in vegetarian food after 14 days of storage, due to treatment with nisin and sodium benzoate (Fang and Chen, 1997). Similar results were obtained by Molinos et al. (2008a) using various chemical compounds in combination with enterocin AS-48 to inactivate *S. enterica* and several of them, such as lactic acid, poliphosphoric acid and peracetic acid, sodium hypochlorite, hexadecylpyridinium chloride, hidrocynnamic acid and 2-propanol, reduced significantly the counts of cells after 48 h storage in soy sprouts. Molinos et al. (2008b) evaluated the effect of combined enterocin AS-48 with cinnamic, hydrocinnamic, polyphosphoric and peracetic acids, cavacrol, sodium hydrochloride and hexadecylpyridinium chloride and observed that the number of viable cells of *B. cereus* in soy sprouts treated with combined solutions was significantly lower in times of 0 and 24 h.

The surfactants are agents which, by their chemical amphipathic characteristics, present the ability to reduce surface tension of liquid interface and may be used as emulsifiers and detergents (Georgiou et al., 1992). Because they present an amphoteric character, they can act expanding the efficiency of sanitizers such as chlorine and peracetic acid (Bastos et al., 2005). The reduction of surface tension can favor interaction of compounds with food surface favoring elimination of microbiota (Bastos et al., 2005). Beuchat and Scouten (2004) evaluated the influence of Tween 80 in the viability of *Salmonella* Poona and observed that this surfactant did not affect adversely the viability of this pathogen. In the literature, no studies were found to correlate with the action of bacteriocins with surfactants, but our results show an increase in the efficiency of bacteriocin in the reduction of growth rate of the microorganism.

These data demonstrate the possibility of using bovicin HC5 for controlling Gram-negative bacteria as long as associated to a specific treatment that disrupts the outer membrane.

Conclusions

Bovicin HC5 associated with membrane destabilizing agents inhibited the growth of *S.* Typhimurium. This observation suggests that bovicin HC5 can be used to inhibit the growth of Gram-negative bacteria as long as associated with some a destabilizing agent of the outer membrane such as EDTA, diethanolamine, gluconic acid, sodium citrate, lactic acid and nitril-tri-acetic acid. However, further studies are needed to investigate the effect of this bacteriocin in food matrices.

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Full Length Research Paper

Early plant growth promotion of maize by various sulfur oxidizing bacteria that uses different thiosulfate oxidation pathway

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The objective of the work was to evaluate the plant growth promotion of maize by thiosulfate-oxidizing bacteria possessing the tetrathionate intermediate (S4I) and/or paracoccus sulfur oxidation (PSO) pathway for thiosulfate oxidation. *Halothiobacillus* sp. possessing the S4I and PSO pathways for thiosulfate oxidation recorded the highest phosphate solubilization (480 µg ml^{-1}), followed by other S4I pathway bearing bacteria (*Microbacterium phyllosphaerae* and *Pandoraea sputorum*). All the tested bacterial strains such as *Halothiobacillus* sp., *Dyella thiooxydans*, *M. phyllosphaerae, Pandoraea sputorum*, and *Pandoraea* sp., consumed the glucose and thiosulfate simultaneously, indicating that these organisms had a mixotrophic metabolism. Results of growth chamber study revealed that inoculation of *D. thiooxydans* and *M. phyllosphaerae* (both holding the S4I pathway) significantly enhanced the maize root length (73 and 67%, respectively), shoot length (27 and 31%), and shoot biomass (58 and 45%), along with the nutrient uptake of P, K, S, Mn, Ca, Cu and Na. Therefore, it was concluded that *D. thiooxydans* and *M. phyllosphaerae* possessing the S4I pathway are effective early plant growth promoting rhizobacteria for maize when compared with other bacteria possessing the S4I and PSO pathway for thiosulfate oxidation.

Key words: Maize, mixotrophic growth, paracoccus sulfur oxidation pathway, phosphate solubilization, tetrathionate intermediate pathway, sulfur nutrition.

INTRODUCTION

As sulfur (S) is an essential nutrient for plant growth, sulfur-deficient conditions causes severe losses in crop yield. Yet, sulfur nutrition has received little attention for decades, since atmospheric inputs have provided

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Abbreviations: S, Sulfur; **S4I,** S4 intermediate pathway; **PSO,** paracoccus sulfur oxidation; **MST,** mineral salt thiosulfate; **TCP,** tri calcium phosphate.

Table 1. Details of bacterial strains with their thiosulfate oxidation pathway and nutritional type (Anandham et al., 2008b).

Classification of the strains based on carbon source utilization	Bacterial strains	Source[c]	NCBI accession No.	Presence of *soxB*	Thiosulfate oxidation pathway
Obligate chemolithoautotroph	*Halothiobacillus* sp. (ATSB2)[a]	*Oryza sativa* ssp. *Japonica*	EF397569	+	S4I and PSO
Facultative chemolithoautotroph	*Dyella thiooxydans* (ATSB10)[b]	*Helianthus annus*	EF397574	-	S4I
Facultative chemolithoautotroph	*Microbacterium phyllosphaerae* (ATSB31) [b]	*Glycine max*	EF397588	-	S4I
Facultative chemolithoautotroph	*Pandoraea* sp. (ATSB30)[b]	*Glycine max*	EF397587	+	S4I and PSO
Facultative chemolithoautotroph	*Pandoraea sputorum* (ATSB28)[b]	*Capsicum annum*	EF397586	-	S4I

[a]Strain could grow only chemolithoautotrophically with reduced sulfur compounds (obligate chemolithoautotroph). [b]Bacterial strains could grow chemolithoautotrophically, chemoorganotrophically and heterotrophically (facultative chemolithoautotroph). [c]Bacteria were isolated from rhizosphere soils of plants cultivated at fields, located in Kimphae, Jung ha- dong (E 128° 49'- 128° 56', N 35°10'- 35° 17'), Kyung Nam Province of Republic of Korea. All the tested organisms could use thiosulfate, tetrathionate, sulfur, sulfite, sulfide and trithionate. +, Positive; -, negative; S4I, tetrathionate intermediate pathway; PSO, paracoccus sulfur oxidation pathway.

adequate amounts. However, recent reductions in sulfur inputs from atmospheric depositions have resulted in a negative sulfur balance in arable soils, making crop plants increasingly dependent on the soil for the sulfur requirement (Kertesz and Mirleau, 2004). Thus, to alleviate this deficiency, sulfur fertilizers are invariably added to soils, usually in a reduced form, such as elemental sulfur. Yet, reduced form of S in the fertilizers must be oxidized by bacteria to sulfate (available form of S) (Wainright, 1984; Grayston and Germida, 1991; Scherer, 2001). In a previous study, the combined application of elemental sulfur and rock phosphate (RP) significantly increased the available P when inoculated with *Acidithiobacillus* sp. It oxidized elemental sulfur into sulfuric acid, which has in turn promoted RP solubilization and also improved the plant growth (Stamford et al., 2007). The inoculation of Thiobacilli with sulfur and whey has also been found to enhance the solubilization of RP (Ghani et al, 1994). In another study, the rate of phosphate solubilization was increased based on the glucose concentration and type of phosphate solubilizing bacteria used (Son et al., 2006; Anandham et al., 2007b).

Two major biochemical pathways for sulfur oxidation have been identified in sulfur oxidizers (Kelly et al., 1997). The first is the 'S4 intermediate' pathway (S4I), which includes the oxidation of tetrathionate or trithionate or polythionate and sulfur from thiosulfate, while the second is the 'paracoccus sulfur oxidation' (PSO) pathway that directly oxidizes thiosulfate into sulfate (Kelly et al., 1997). In the PSO pathway, the thiosulfate oxidation is carried out by a thiosulfate-oxidizing-multi-enzyme system (TOMES), where sulfate thiol esterase or sulfate thiol hydrolase is coded by *soxB*, which contains a prosthetic manganese cluster in the reaction center, and is essential for thiosulfate oxidation, by *Paracoccus pantotrophus* (Friedrich et al. 2001). The existence of more than one thiosulfate oxidation system within a single bacterium has already been documented (Hensen et al., 2006; Anandham et al., 2008b).

While several studies have reported on the enhancement of sulfur availability, rock phosphate solubilization, and the plant growth promotion of sulfur-oxidizing bacteria (Grayston and Germida, 1991; Stamford et al., 2002; El-Tarabily et al., 2006; Anandham et al., 2007a, 2008a), none of these studies have correlated the thiosulfate oxidation pathway with sulfur oxidation and plant growth promotion. In a previous study, several thiosulfate-oxidizing bacteria were isolated from the rhizosphere of crop plants and documented their thiosulfate oxidation pathway (Anandham et al., 2008b). Accordingly, this study examined the solubilization of tri-calcium phosphate resulting from the oxidation of thiosulfate and early plant growth promotion of maize when inoculating thiosulfate-oxidizing bacteria possessing the S4I and/or PSO pathway for thiosulfate oxidation.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bacterial strains used in this study and their mode of thiosulfate oxidation are listed in Table 1. Unless otherwise stated, all the bacterial strains were cultivated in a mineral salts thiosulfate (MST) medium containing (gl^{-1}): NH_4Cl, 1.0; K_2HPO_4, 4.0; KH_2PO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 0.5, $Na_2S_2O_3 \cdot 5H_2O$, 5.0; yeast extract, 0.05; and trace element solution 5 ml; pH 6.5 at 30°C for 4 days (Mukhopadhyaya et al., 2000).

Tri-calcium phosphate (TCP) solubilization

The TCP solubilization ability of the bacteria with different thio-

sulfate oxidation pathways was examined in a slightly modified Waksman and Joffe medium that contained (gl^{-1}); $Na_2S_2O_3 \cdot 5H_2O$, 5.0; $(NH_4)_2SO_4$, 2.0; $MgSO_4$, 0.5; $FeSO_4$, 0.01; KH_2PO_4, 5.0; TCP, 5.0; and glucose (0, 5.0 and 10.0) (Waksman and Joffe, 1922). A quantitative assessment of the solubilization was carried out in Erlenmeyer flasks (250 ml) containing 100 ml of the liquid medium inoculated with 1 ml of the thiosulfate-oxidizing bacteria (1 x 10^7 cfu ml^{-1}) and incubated at 30°C under constant shaking (120 rpm). An autoclaved uninoculated medium served as the control. The soluble P content in the culture supernatant was assayed using the method of Murphy and Riley (1962). The thiosulfate consumption was assayed spectrophotometrically using a cyanolytic method (Kelly and Wood, 1994), and the glucose concentration in the growth medium was assayed according to the method reported by Nelson (1944).

Determination of growth and nutritional parameters

This experiment was conducted to determine the potential of thiosulfate-oxidizing bacteria with various biochemical pathways for thiosulfate oxidation to stimulate maize growth by increasing the plant-available sulfate and phosphate (through rock phosphate solubilization) in the rhizosphere through sulfur oxidation. The bacterial strains were grown in the MST medium, harvested by centrifugation, washed twice, and suspended in saline (0.85% Nacl). Meanwhile, the maize seeds (*Zea mays*) (Hungnong Seed Co. Ltd., Korea and Seminis Korea Inc., Korea) were surface disinfected in 70% ethanol for 1 min, immersed in 0.5% NaOCl for 2 min, and washed 4 times with sterilized distilled water. The surface-sterilized seeds were then shaken in the bacterial suspension (1 x10^7 cfu ml^{-1}) for 2 h, axenically dried seeds were placed in a Petri dish with moist sterile filter paper, and incubated for 7 days at 25 ± 1°C under darkness. On day 7, the germinated maize seeds were transplanted into a pot containing acid-washed autoclaved sand (350 g) amended with elemental sulfur (50 µg g^{-1} of sand) as the sulfur source. Two milliliters (1 x10^7 cfu ml^{-1}) of the different bacterial strains was also placed in a 2-cm hole made in the pots before transplanting. Control treatment consists of pre-germinated maize seeds that had been previously soaked in saline to aid germination. It was transplanted into sand either unamended with sulfur or amended with Na_2SO_4 (50 µg SO_4-S g^{-1} of sand). The control without sulfur was included to determine the growth of the maize under sulfur-deficient conditions, whereas the control with Na_2SO_4 as the sulfur source were included to determine the growth of the maize in the presence of readily available sulfate and the treatment details are given in Table 5. For each treatment, 30 pots were maintained, each with a single plant, were arranged in a completely randomized block design with three replications. The pots were placed in a growth chamber at 25 ± 1°C with a photoperiod of 12 h dark followed by 12 h light (18 µmol m^{-2} S^{-1}). The plants were watered regularly with sterile distilled water. Each pot received the following basal nutrients: 68 mg N [26 mg NH_4NO_3, 28 mg $Ca(NO_3)_2 \cdot 4H_2O$, and 14 mg Mg $(NO_3)_2 \cdot 6H_2O$]; 10 mg P as K_2HPO_4 or 83.3 mg RP that supplies 10 mg P; 160 mg Ca as Ca $(NO_3)_2 \cdot 4H_2O$; 48 mg Mg as $Mg(NO_3)_2 \cdot 6H_2O$; 1 mg Mn as $MnCl_2 \cdot 4H_2O$; 0.2 mg Zn as $Zn(C_2H_3O_2)_2$; 0.01 mg Cu as $CuCl_2 \cdot 2H_2O$; 2 mg Fe as Fe-EDTA; and 0.005 mg Mo as $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (Histuda et al., 2005). After 30 days, the plants were harvested to determine the root and shoot length and dry weight. The nutrient contents in the plant material were also analyzed. The total nitrogen (Kjeldahl) was determined as per the procedures outlined by Page et al. (1982). After nitric and perchloric acid digestion of the plant samples, the P, K, Mg, Mn, Cu, Ca, Na, Zn, and Fe were determined using an ICP-OES (Optima 5300DV,

Perkin Elmer, USA). Finally, the total sulfur content in the plant samples was determined using a turbidometric analysis after wet oxidation with magnesium nitrate and perchloric acid (Nes, 1979).

Statistical analysis

The data were analyzed by an analysis of variance (ANOVA) using the general linear model version 9.1; SAS institute Inc, Cary, NC, USA. The means were compared using the least significant difference (LSD). The significance levels were within confidence limits of 0.05 or less.

RESULTS

TCP solubilization

The inoculation of the thiosulfate-oxidizing bacteria significantly enhanced the TCP solubilization, and the soluble P level in the Waksman and Joffe medium increased with the incubation time (Table 2). Statistical analysis revealed maximum thiosulfate consumption and TCP solubilization in the medium unamended with glucose, followed by the media containing 0.5 and 1.0% glucose (Tables 2 and 3). The supplied (20 mM) thiosulfate was almost completely consumed in the medium devoid of glucose and hence the experiment was terminated on day 18 (Table 3). The medium amended with thiosulfate and glucose and inoculated with the thiosulfate-oxidizing bacteria utilized both the thiosulfate and glucose (Tables 3 and 4). Among the tested bacterial strains, *Halothiobacillus* sp. with the S4I and PSO pathways consumed the maximum thiosulfate (19.89 mM) and showed the highest soluble P in the growth medium devoid of glucose (480.49 µg P ml^{-1}) (Tables 2 and 3). Similarly on day 18, the facultative chemolithoautotrophic *Pandoraea sputorum* (possessing the S4I pathway for thiosulfate oxidation) inoculated into the medium containing 20 mM of thiosulfate plus 0.5 or 1% glucose recorded the highest soluble P, followed by *Halothiobacillus* sp. and *Pandoraea* sp. (including the S4I + PSO pathways) (Table 2).

Early plant growth promotion in maize

Five thiosulfate-oxidizing bacteria with different thiosulfate oxidation pathways were tested for their potential to promote maize growth. *Halothiobacillus* sp. with the S4I and PSO pathways had no effect on the early maize growth. However, the growth was stimulated when sulfur was added to the sand in either reduced form (elemental sulfur) with the inoculated bacteria or oxidized form (Na_2SO_4). In this experiment, sulfur-deficiency symptoms (yellowing of young leafs) were noticed in the plants in the control treatment. Meanwhile, *D. thiooxydans* and *M. phyllosphaerae* with the S4I pathway significantly enhanced the root length (73 and 67%, respectively), shoot length (27 and 31%), and shoot biomass (58 and 45%) when compared with the uninoculated control (Table 5). Also, *Pandoraea* sp. and *Pandoraea sputorum* significantly enhanced the plant biomass (Table 5). However, the plant growth promotion effect was not significant for the treatment amended with RP as compared to the treatment supplied with readily available

Table 2. Tri-alcium phosphate solubilization by thiosulfate oxidizing bacteria.

Bacterial strains/ growth condition	Soluble P (µg ml^{-1})		
	Incubation day		
	6	12	18
No Glucose			
Halothiobacillus sp.	86.88 ± 4^{a}	207.15 ± 2^{a}	480.49 ± 5^{a}
Dyella thiooxydans	75.41 ± 2^{b}	195.77 ± 2^{cd}	405.28 ± 6^{f}
M. phyllosphaerae	69.47 ±6^{c}	205.53 ± 4^{ab}	422.56 ± 4^{c}
Pandoraea sp.	65.65 ± 3^{d}	203.09 ± 5^{ab}	429.67 ± 9^{b}
Pandoraea sputorum	65.22 ± 5^{d}	186.83 ± 6^{efg}	417.48 ± 7de
0.5% Glucose			
Halothiobacillus sp.	74.14 ± 5^{b}	199.84 ± 9^{bc}	420.53 ± 10^{cd}
Dyella thiooxydans	66.92 ± 3^{cd}	185.20 ± 5^{gh}	405.28 ± 5^{f}
M. phyllosphaerae	66.50 ± 5^{d}	205.53 ± 4^{ab}	404.27 ± 4^{f}
Pandoraea sp.	65.65 ± 2^{d}	195.77 ± 5^{cd}	418.50 ±6^{fde}
Pandoraea sputorum	66.07 ± 8^{d}	181.95 ± 3^{ghf}	430.69 ± 5^{b}
1.0% Glucose			
Halothiobacillus sp.	66.89 ± 3^{cd}	191.71 ± 6^{def}	406.30 ± 6^{f}
Dyella thiooxydans	64.37 ± 4^{d}	173.82 ± 3^{j}	379.88 ± 9^{g}
M. phyllosphaerae	65.65 ± 5^{d}	192.52 ± 2de	403.25 ± 3^{f}
Pandoraea sp.	60.98 ± 2^{e}	174.63 ± 4^{ij}	416.46 ± 4^{e}
Pandoraea sputorum	59.70 ± 2^{e}	180.33 ± 9^{ih}	420.53 ± 5^{cd}
LSD ($P \leq 0.05$)	2.93	2.71	3.86

Tri-calcium phosphate solubilization of thiosulfate oxidizing bacteria due to production of sulfuric acid while oxidation of thiosulfate in Waksman and Joffe medium amended with or without glucose were determined. Values are the mean of three replications ± SD. Values in each column followed by same letter (s) are not significantly different.

Table 3. Thiosulfate consumption by thiosulfate oxidizing bacteria.

Bacterial strains/ growth condition	Thiosulfate consumed (mM)		
	Incubation day		
	6	12	18
No Glucose			
Halothiobacillus sp.	11.07 ± 1^{abc}	18.40 ± 0.4^{a}	19.89 ± 0.9^{a}
Dyella thiooxydans	9.25 ± 0.5^{de}	12.09 ± 1^{cde}	16.32 ± 0.52^{d}
M. phyllosphaerae	10.33 ± 0.83^{cde}	13.32 ± 0.82^{c}	15.67 ± 0.87^{de}
Pandoraea sp.	10.33 ± 1^{cde}	16.24 ±0.49^{b}	19.02 ± 0.55^{abc}
Pandoraea sputorum	12.46 ± 0.96^{a}	17.80 ± 1.48^{a}	19.99 ± 0.09^{a}
0.5% Glucose			
Halothiobacillus sp.	10.72 ± 1^{bcd}	17.55 ± 1^{ab}	19.11 ± 0.27^{abc}
Dyella thiooxydans	9.05 ± 0.46^{e}	11.20 ± 0.7^{ef}	15.85 ± 1de
M. phyllosphaerae	10.12 ± 0.62^{cde}	11.74 ± 0.84^{def}	14.93 ± 1.43^{ef}
Pandoraea sp.	10.84 ± 1^{abcd}	11.10 ± 0.6^{ef}	18.15 ± 0.25^{c}
Pandoraea sputorum	12.21 ± 0.46^{abc}	16.90 ± 1^{ab}	19.61 ± 0.29^{ab}
1.0% Glucose			
Halothiobacillus sp.	10.10 ± 1^{cde}	17.00 ± 1^{ab}	18.55 ± 1b^{c}

Table 3. Contd.

Dyella thiooxydans	8.81 ± 1.01^{e}	10.01 ± 0.51^{f}	15.69 ± 0.94^{de}
M. phyllosphaerae	9.88 ± 0.5^{cde}	10.72 ± 1^{ef}	13.94 ± 1^{f}
Pandoraea sp.	11.32 ± 0.8^{abc}	10.51± 1.04^{f}	14.42 ± 0.92^{f}
Pandoraea sputorum	12.05 ± 1^{ab}	12.86 ± 1^{cd}	16.64 ± 0.74^{f}
LSD ($P \leq 0.05$)	1.64	1.50	1.24

Thiosulfate consumption of thiosulfate oxidizing bacteria in Waksman and Joffe medium amended with and without glucose. Values are the mean of three replications ± SD. Values in each column followed by same letter (s) are not significantly different.

Table 4. Glucose consumption by thiosulfate oxidizing bacteria.

Bacterial strains/ growth condition	Glucose consumed (mg 100 ml^{-1})	
	Incubation day	
	6	18
0.5% Glucose		
Halothiobacillus sp.	0.397 ± 0.07^{e}	0.444 ± 0.04^{e}
Dyella thiooxydans	0.426 ± 0.03^{de}	0.464 ± 0.02^{d}
M. phyllosphaerae	0.415 ± 0.01^{e}	0.485 ± 0.02^{c}
Pandoraea sp.	0.481 ± 0.05^{c}	0.484 ± 0.03^{c}
Pandoraea sputorum	0.453 ± 0.03^{cd}	0.495 ± 0.01^{c}
1.0% Glucose		
Halothiobacillus sp.	0.874 ± 0.03^{b}	0.938 ± 0.02^{a}
Dyella thiooxydans	0.897 ± 0.04^{ab}	0.904 ± 0.02^{b}
M. phyllosphaerae	0.888 ± 0.02^{ab}	0.909 ± 0.01^{b}
Pandoraea sp.	0.915 ± 0.02^{a}	0.931 ± 0.02^{a}
Pandoraea sputorum	0.901 ± 0.02^{ab}	0.903 ± 0.02^{b}
LSD ($P \leq 0.05$)	0.03	0.02

Glucose consumption of thiosulfate oxidizing bacteria in Waksman and Joffe medium amended with glucose 0.5 and 1% glucose. Values are the mean of three replications ± SD. Values in each column followed by same letter (s) are not significantly different.

Table 5. Effectct of thiosulfate oxidizing bacterial inoculation on biomass production of maize plant on 30 days after planting.

Treatment	Root Length (cm)	Shoot Length (cm)	Root Biomass (mg plant^{-1})	Shoot Biomass (mg plant $^{-1}$)
Halothiobacillus sp.+S^0	8.3 ± 2.2^{cd}	28.8 ± 2.0^{gf}	50 ± 2.0^{f}	192 ± 1.0^{l}
D. thiooxydans +S^0	14.6 ± 2.1^{a}	36.1 ± 1.0^{a}	183 ± 5.0^{a}	346 ± 3.0^{a}
M. phyllosphaerae+S^0	14.1 ± 2.7^{ab}	37.3 ± 3.0^{a}	180 ± 2.0^{a}	318 ± 2.0^{c}
Pandoraea sp.+S^0	7.5 ± 2.3^{d}	34.1 ± 1.0^{b}	120 ± 2.0^{bc}	279 ± 3.0^{d}
P. sputorum+S^0	11.5 ± 2.4^{abc}	27.8 ± 2.0^{g}	72 ± 1.0^{ef}	230 ± 2.0^{h}
Halothiobacillus sp.+S^0+RP	8.6 ± 1.3^{cd}	28.3 ± 2.0^{g}	82 ± 2.0^{de}	224 ± 2.0^{l}
D. thiooxydans +S^0+RP	11.0 ± 1.0^{bcd}	33.3 ± 3.0^{bc}	101 ± 4.0^{cd}	246 ± 2.0^{f}
M. phyllosphaerae +S^0+RP	6.5 ± 1.8^{cd}	27.4 ± 3.0^{g}	60 ± 2.0^{ef}	260 ± 2.0^{e}
Pandoraea sp.+S^0+RP	9.0 ± 1.6^{cd}	30.6 ± 2.0^{de}	58 ± 3.0^{ef}	237 ± 3.0^{g}
P. sputorum +S^0+RP	6.5 ± 0.4^{cd}	30.1 ± 2.0^{ef}	76 ± 1.0^{def}	229 ± 2.0^{h}
S^0+RP	14.6 ± 1.3^{a}	32.0 ± 1.0^{cd}	78 ± 2.0^{de}	222 ± 1.0^{j}
Na_2SO_4	11.8 ± 1.7^{abc}	36.9 ± 4.0^{a}	142 ± 6.0^{b}	329 ± 2.0^{b}
Control	8.5 ± 0.3^{cd}	28.5 ± 3.0^{g}	50 ± 4.0^{f}	219 ± 1.0^{k}
LSD ($P \leq 0.05$)	3.6	1.6	25.2	2.8

RP, rock phosphate; S^0, elemental sulfur. Values in each column are the mean of three replications of ± SD. Values in each column followed by the same letter (s) are not significantly different.

Table 6. Effect of thiosulfate oxidizing bacteria inoculation on macro and secondary nutrient uptake of maize plant on 30 days after planting.

Treatment	N (%)	P	K	S
		(µg g^{-1} plant tissue)		
Halothiobacillus sp.+S^0	4.22 ± 0.02^{ijk}	35.02 ± 0.15^{c}	21.28 ± 0.25^{j}	3.84 ± 0.06^{k}
D. thiooxydans +S^0	4.11 ± 0.11^{k}	42.65 ± 0.30^{a}	21.67 ± 0.55^{i}	12.17 ± 0.15^{a}
M. phyllosphaerae+S^0	5.27 ± 0.07^{a}	35.27 ± 0.27^{c}	26.79 ± 0.45^{d}	7.64 ± 0.05^{e}
Pandoraea sp.+S^0	4.71 ± 0.03^{de}	35.96 ± 0.90^{b}	21.27 ± 0.27^{j}	6.64 ± 0.04^{hg}
P. sputorum+S^0	4.33 ± .03^{hijk}	31.00 ± 0.50^{e}	22.21 ± 0.21^{hg}	9.13 ± 0^{c}
Halothiobacillus sp.+S^0+RP	4.72 ± 0.04^{de}	21.13 ± 0.13^{l}	21.93 ± 0.60^{ih}	4.90 ± 0.26^{j}
D. thiooxydans +S^0+RP	4.43 ± 0.04^{ghij}	27.79 ± 0.24^{h}	30.71 ± 0.33^{b}	9.55 ± 0.04^{b}
M. phyllosphaerae +S^0+RP	4.68 ± 0.05^{def}	28.45 ± 0.45^{g}	22.47 ± 0.11^{g}	6.20 ± 0.21^{i}
Pandoraea sp.+S^0+RP	5.12 ± 0.12^{ab}	22.43 ± 0.30^{k}	24.62 ± 0.09^{f}	8.68 ± 0.07^{d}
P. sputorum +S^0+RP	4.29 ± 0.17^{ijk}	24.77 ± 0.26^{j}	22.01 ± 0.10^{ih}	7.21 ± 0.16^{f}
S^0+RP	4.57 ± 0.05^{defg}	26.87 ± 0.50^{i}	31.05 ± 0.17^{b}	6.63 ± 0.11^{g}
Na_2SO_4	4.46 ± 0.03^{fghi}	35.07 ± 0.42^{c}	32.90 ± 0.95^{a}	6.43 ± 0.07^{hi}
Control	4.79 ± 0.29^{cd}	30.15 ± 0.15^{f}	29.02 ± 0.24^{c}	0.10 ± 0.08^{l}
LSD ($P \leq 0.05$)	0.23	0.34	0.39	0.06 ± 0.08

RP, rock phosphate; S^0, elemental sulfur. Values in each column are the mean of three replications of ± SD. Values in each column followed by the same letter (s) are not significantly different.

P. The analysis of the nutrient contents in plant tissues revealed that the thiosulfate-oxidizing bacterial inoculation significantly improved the nutrient uptake. The *D. thiooxydans* inoculated plants showed a significant increase in the nutrient uptake including P, K, S, Mn, Ca, Cu, and Na, followed by *M. phyllosphaerae* (Tables 6 and 7). *Pandoraea* sp. and *Pandoraea sputorum* inoculated plants significantly improved the plant uptake of P, S, Mn, Zn and Na as compared to control (Tables 6 and 7).

DISCUSSION

Thiosulfate-oxidizing bacteria are known to have at least two thiosulfate oxidation pathways for oxidizing thiosulfate into sulfate. Besides, the existence of a common mechanism for thiosulfate oxidation in all thiosulfate-oxidizing bacteria has also been proposed (Kelly et al., 1997; Friedrich et al., 2001). However, this study demonstrated the effect of sulfur oxidizing bacteria possessing different thiosulfate oxidation pathway on TCP solubilization and early plant growth promotion of maize. Thiosulfate was used to test the lithotrophic process since; thiosulfate is used as electron donor by most of the sulfur oxidezing bacteria including chemolithotrophs and chemoheterotrophs (Mukhopadhyaya et al., 2000). In the present study, chemolithoautotrophic *Halothiobacillus* sp. consumed highest thiosulfate as compared to other strains. Earlier studies have also reported that chemolithoautotrophic thiosulfate oxidizers consumed the maximum thiosulfate as compared to other facultative chemolithoautotrophs and chemoheterotrophs (Kelly et al., 1997; Sievert et al., 2000).

High phosphate sorption in soils is a serious limiting factor for plant productivity and phosphate fertilization efficiency. In the current study, TCP solubilization due to the oxidation of thiosulfate into sulfuric acid by thiosulfate-oxidizing bacteria was examined in a medium amended with and without glucose. The TCP solubilization only increased in the medium devoid of glucose, which can be explained by the fact that the prolonged metabolic state of the cells with the increased substrate (glucose) repressed the formation of the enzyme or enzymes responsible for thiosulfate oxidation (Pepper and Miller, 1978; Wood et al., 2004). In the present study, the process of preparing the partially acidulated rock phosphate was also found to take a relatively long time when the RP and sulfur mixture was amended with organic substrates and inoculated with chemolithotrophic thiosulfate-oxidizing bacteria. However, in previous studies, amendments with whey and glucose were found to increase the RP and TCP solubilization (Ghani et al., 1994; Son et al., 2006; Anandham et al., 2007b), yet these studies used heterotrophic thiobacilli and heterotrophic phosphate-solubilizing bacteria, which could explain the anomaly. *Halothiobacillus* sp. possessing the S4I and PSO pathways for thiosulfate oxidation recorded the highest soluble P, whereas the other S4I pathway bacteria (*M. phyllosphaerae* and *Pandoraea sputorum*) and S4I + PSO pathway bacterium (*Pandoraea* sp.) were statistically at par with each other. In this study, all the tested strains consumed the glucose and thiosulfate, indicating that these bacteria have a mixotrophic metabolism (Anandham et al., 2007c). Mixotrophic growth (the utilization of organic and inorganic substrates) may in fact be metabolically advantageous for these bacteria. Since low

Table 7. Effect of thiosulfate oxidizing bacteria inoculation on micro nutrient uptake of maize plant on 30 days after planting.

Treatment	(μg g^{-1} plant tissue)				(mg kg^{-1} plant tissue)		
	Fe	Mg	Mn	Zn	Ca	Cu	Na
Halothiobacillus sp.+S^0	1.17 ± 0.10^{i}	3.54 ± 0.28^{f}	0..40 ± 0.02^{c}	0.872 ± 0.002^{b}	6.94 ± 0.25^{f}	21.74 ± 0.5^{m}	0.291 ± 0.003^{k}
D. thiooxydans +S^0	2.41 ± 0.30^{cd}	5.45 ± 0.20^{a}	0.71 ± 0.03^{a}	0.102 ± 0.001^{f}	10.56 ± 0.56^{a}	124.38 ± 0.60^{a}	0.951 ± 0.028^{a}
M. phyllosphaerae+S^0	1.36 ± 0.33^{ih}	4.70 ± 0.30^{b}	0.46 ± 0.06^{b}	0.167 ± 0.003^{d}	8.38 ± 0.38^{d}	121.72 ± 0.72^{b}	0.829 ± 0.002^{d}
Pandoraea sp.+S^0	1.54 ± 0.25^{gh}	4.11 ± 0.11^{de}	0.44 ± 0.04^{b}	0.046 ± 0.001^{g}	5.79 ± 0.45^{g}	41.9 ± 0.45^{h}	0.756 ± 0.005^{f}
P. sputorum+S^0	1.44 ± 0.22^{h}	4.11 ± 0.15^{de}	0.29 ± 0.04^{d}	0.019 ± 0.001^{i}	5.78 ± 0.23^{g}	27.36 ± 0.26^{l}	0.471 ± 0.001^{i}
Halothiobacillus sp.+S^0+RP	3.17 ± 0.14^{b}	4.21 ± 0.15^{d}	0.40 ± 0.05^{c}	0.104 ± 0.003^{f}	6.78 ± 0.50^{f}	44.07 ± 0.23^{f}	0.794 ± 0.004^{e}
D. thiooxydans +S^0+RP	3.60 ± 0.30^{a}	4.13 ± 0.17^{d}	0.22 ± 0.02^{e}	0.953 ± 0.003^{a}	8.62 ± 0.55^{cd}	47.97 ± 0.28^{d}	0.894 ± 0.002^{b}
M. phyllosphaerae +S^0+RP	2.55 ± 0.55^{c}	3.91 ± 0.24^{e}	0.12 ± 0.01^{f}	0.103 ± 0.002^{f}	9.26 ± 0.13^{b}	45.92 ± 0.46^{e}	0.170 ± 0.002^{m}
Pandoraea sp.+S^0+RP	2.01 ± 0.12^{e}	4.15 ± 0.15^{d}	0.47 ± 0.02^{b}	0.041 ± 0.003^{h}	10.53 ± 0.27^{a}	32.11 ± 0.11^{j}	0.656 ± 0.006^{g}
P. sputorum +S^0+RP	1.72 ± 0.26^{gf}	3.20 ± 0.20^{g}	0.14 ± 0.01^{f}	0.205 ± 0.005^{c}	7.69 ± 0.25^{e}	28.61 ± 0.42^{k}	0.396 ± 0.003^{j}
S^0+RP	2.05 0.11^{e}	4.15 ± 0.15^{d}	0.19 ± 0.02^{e}	0.011 ± 0.001^{k}	8.31 ± 0.26^{d}	34.50 ± 0.50^{i}	0.222 ± 0.001^{l}
Na_2SO_4	2.43 ± 0.43^{cd}	4.45 ± 0.45^{c}	0.22 ± 0.02^{e}	0.126 ± 0.006^{e}	7.14 ± 0.14^{f}	43.22 ± 0.22^{g}	0.856 ± 0.003^{c}
Control	2.29 ± 0.10^{d}	5.74 ± 0.13^{a}	0.03 ± 0^{g}	0.001 ± 0l	10.46 ± 0.38^{a}	47.89 ± 0.25^{d}	0.174 ± 0.002^{m}
LSD ($P \leq 0.05$)	0.22	0.20	0.03	0.003	0.36	0.66	0.34

RP, rock phosphate; S^0, elemental sulfur. Values in each column are the mean of three replications of ± SD. Values in each column followed by the same letter (s) are not significantly different.

concentrations of sulfur compounds can limit growth, the usage of organic carbon for biomass synthesis or even the co-oxidation of sulfur compounds together with organic substrates may ensure the growth and better survival of sulfur-oxidizing bacteria in the rhizosphere (Anandham et al., 2007c). To the best of our knowledge, this is the first report on the mixotrophic growth of *D. thiooxydans*, *M. phyllosphaerae*, and *Pandoraea* spp.

The effect of thiosulfate-oxidizing bacteria on sulfur nutrition during the early growth of maize was tested since sulfur-deficiency symptoms usually occur during the early stages of crop growth and may disappear later. However, sulfur deficiency symptom could easily be noted in maize plant, hence it is considered as indicator plant for assessing the sulfur deficiency of soils (Grayston and Germida, 1991). Inoculation experiments with microbes initially are performed under gnotobiotic condition in sand to assess their colonization potential (Egamberdieva, 2010). Hence, in the current study, plant growth promoting potential of thiosulfate oxidizing bacteria was assessed in sand under gnotobiotic condition. Previous studies reported the plant growth promotion of wheat and maize were assessed through inoculation of *Pseudomonas* sp. and nitrogen fixers, respectively in sand under gnotobiotic condition (Mehnaz and Lazarovits, 2006; Egamberdieva, 2010). In the growth chamber study, sulfur is normally used instead of thiosulfate, as it is considered an effective fertilizer or soil acidulant in a wide range of agricultural soils (Janzen and Bettany, 1987). The application of sulfur and/or inoculation of the thiosulfate-oxidizing bacteria enhanced the shoot and root length and the plant biomass of the maize. Several earlier reports have also noted the beneficial effect of sulfur application for the crop growth of groundnut, canola and maize (Hago and Salama, 1987; Blake-Kalff et al., 1998; Histuda et al., 2005; El-Tarabily et al., 2006). Deficiency symptom was also observed with the control treatments, where all the nutrients, except for sulfur, were amended, thereby implying that the application of NPK alone is not sufficient for overall plant growth. While the sulfur oxidation potential of *Halothiobacillus* sp. has already been well documented under laboratory conditions, the inoculation of *Halothiobacillus* sp. in this study did not promote plant growth (Anandham et al., 2008a, 2008b). For the plants treated with the S4I pathway bacteria (*D. thiooxydans* and *M. phyllosphaerae*), the root length was longer and the root biomass was

greater than the treatment in which plants that received readily available sulfate. This may be due to the fact that these bacteria in addition to S oxidation also have various plant growth promoting traits that were previously demonstrated (Anandham et al., 2008a). Thus, these two bacteria may have enhanced the maize growth via two (or more) mechanisms simultaneously. The plant growth promotion effect was not significant for the treatment amended with RP as compared to the treatment supplied with readily available P. Since, sulfur is oxidized into thiosulfate and other intermediate products (Grayston and Germida, 1991), and the conversion of these intermediate products into sulfuric acid takes quite a long time, this likely delayed the release of P_2O_5 from the rock phosphate (Janzen and Bettany, 1987; Anandham et al., 2008b). Previously, inoculation of plant growth promoting bacteria in maize significantly improved the maize growth and yield (Gholami et al., 2012).

In addition to increasing the sulfur and nitrogen uptake in maize, the inoculation of *D. thiooxydans* and *M. phyllosphaerae* also increased the uptake of Fe, Mn, Zn, Ca, Cu and Na, probably the inoculated bacteria acidified the microenvironment around the maize roots and solubilized the nutrients ultimately taken up by the plants (Grayston and Germida, 1991). Inoculation of sulfur-oxidizing bacteria increased the availability of phosphorus, iron and zinc in calcareous soils (Heydarnezhad et al., 2012).The thiosulfate-oxidizing bacteria with the S4I pathway for thiosulfate oxidation (*D. thiooxydans* and *M. phyllosphaerae*) increased the soil sulfate content and promoted the maize plant growth as compared to bacteria possessing the S4I and PSO pathways for thiosulfate oxidation. During the process of sulfur oxidation by the thiosulfate-oxidizing bacteria, thiosulfate, trithionate, tetrathionate and polythionates were probably accumulated in the sand. These reduced inorganic sulfur compounds may have then supported the chemolithoautotrophic growth of the bacteria including the S4I pathway for thiosulfate oxidation, thereby enhancing the survivability of the inoculated bacteria and ultimately the maize plant growth. Meanwhile, the lack of plant growth promotion by the inoculated *Halothiobacillus* sp., *Pandoraea* sp. and *P. sputorum* including the S4I and PSO pathways and S4I pathway, respectively, may have been due to a strain-dependant phenomenon or poor colonization potential, however, this needs to be investigated through further studies.

Conclusion

This study demonstrated that the thiosulfate-oxidizing bacteria *D. thiooxydans* and *M. phyllosphaerae* including the S4I pathway were both effective early plant growth promoting rhizobacteria for maize. The findings of the present study will be further investigated by generating transposon insertion mutants with impaired thiosulfate oxidation to study the correlation between the thiosulfate oxidation pathway and sulfur oxidation-mediated plant growth promotion. In addition, the exact mechanism of the maize plant growth promotion by these thiosulfate-oxidizing bacteria will be studied without any sulfur amendment, and in comparison with other plant growth promoting bacteria that lack the sulfur oxidation trait.

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Full Length Research Paper

Synergistic effect of ampicillin and gentamicin on beta-lactamase producing and methicillin resistant staphylococci

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The antibacterial effect of ampicillin, gentamicin, vancomycin, methicillin and other drugs was tested on both beta-lactamase producing and methicillin resistant staphylococci. A total of 60 clinical isolates were collected from Irrua Specialist Teaching Hospital, Irrua. The results reveal that 32 (53.3%) of the staphylococci isolated produced beta-lactamase while 28 (47.7%) did not produce beta-lactamase. The difference in beta-lactamase production by the *Staphylococcus aureus* isolates was not statistically significant (P > 0.05). Out of the 32 beta-lactamase producing species, non was sensitive to methicillin while six of the 28 non beta-lactamase producing strains were sensitive to methicillin. The effects of ampicillin, gentamicin and the synergy of both drugs at different concentration (20, 15, 0.5 and 0.25 µg) did not show good sensitivity except for the 20 µg. A correlation (R < 1) was observed between beta-lactamase production and multi drug resistance, between beta-lactamase production and methicillin resistance and methicillin resistance and multi drug resistance.

Key words: *Staphylococcus aureus* beta-lactamase, antimicrobial resistance.

INTRODUCTION

The three main *Staphylococcus* species of clinical importance are *Staphylococcus aureus, Staphylococcus epidermidis* and *Staphylococcus saprophyticus* (Cruickshank et al., 1975). Staphylococci causes infections either through their ability to multiply and spread on tissues or through their production of many extra-cellular substances like toxins and enzymes for example beta lactamase which are controlled by plasmids or chromosomes born genes (Ito et al., 2004). One of the reasons for the success of the staphylococci as human pathogen is its great variability, occurring at different periods and places with diverse clonal types and antibiotic resistance patterns within regions and countries. Although infections caused by antibiotic-resistant *S. aureus* bring about serious problems in the general population, such infections can be particularly devastating for the very young, the elderly and the immuno-compromised (Adebayor and Lin, 2006).

The beta-lactamase antibiotics include the penicillins cephalosporins, carbapenems, clavams and monobactams (Singleton and Willey, 1999). Beta-lactam antibiotics act by disrupting synthesis of the cell envelope in growing cells. They do this by inactivating penicillin binding proteins thus inhibiting the synthesis of the peptideglycan. In many antibiotics, the beta-lactam ring is susceptible to cleavage by certain bacterial enzymes (B-lactamase) (Edwards and Greenwood, 1992).

Such cleavage destroys the antibiotics and the organism which produces these enzymes show at least some degree of resistance to beta-lactam antibiotics. The

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Table 1. Beta-lactamase production among staphylococci isolates.

Specimen	Number of isolates	Number positive for b-lactamase	Number negative for b-lactamase
Urine	20	14	6
Wound swab	12	6	6
Urethral swab	8	4	4
Sputum	4	1	3
Ear swab	9	6	3
Endocervical swab	5	1	4
Pleural aspired	2	-	2
Total %	60 (100%)	32 (53.3%)	28 (47.7%)

t= 0.15; p>0.05.

production of beta-lactamase in *S. aureus* is specified by *blaZi* genes and is believed to be regulated by a repressor blaZi (Clarks and Duke, 2001). The production of beta-lactamase can be detected by the iodometric or acidometric method (Singleton and Wiley, 1999).

Over the past 20 years, there has been an increased interest in the development of resistance of pathogens against antibiotics caused by the indiscriminate use of modern antibiotic (Dash et al., 2011). Methicillin resistant *S. aureus* (MRSA) is a bacterium that developed antibiotic resistance first to penicillin in 1947 and later to methicillin and related "anti *Staphylococcus* drugs". It was first discovered in Britain in 1961 just one year after the launch of methicillin and is now widespread causing serious hospital and community acquired infections worldwide (Enright, 2003). Antibiotic resistance due to beta-lactamase production possesses a clinical problem which may be approached by the use of drug synergy. This study was aimed at studying the use of combination of drugs therapy staphylococcal infection.

MATERIALS AND METHODS

Specimen collection/ isolation of organism

Specimens used in this study were collected from in and out patients at Irrua Specialist Teaching Hospital Irrua, Edo State, Nigeria. They included urine, ear swab, urethra swab, endocervical swab, sputum and pleural aspirate. A total of 60 clinical isolates was gotten. Samples were inoculated separately into Mannitol salt agar plate using the streak plate method and incubated aerobically at 37°C for 24 h. At the end of incubation, isolates were identified using various morphological and biochemical tests as described by Cheesbrough (2006). The acidometric method was used to identify beta-lactamase production.

Antibacterial susceptibility

The effect of ampicillin, gentamicin, and synergy of both drugs at different concentrations was tested on seven of the isolates and a standard *S. aureus* strain (NCTC 6571). Ampicillin (500 mg/2 ml) was diluted to 20 µg/ml. The isolates were suspended in nutrient broth before subjecting to different concentrations of ampicillin. Gentamicin (80 mg /ml) was diluted to 20 µg /ml. The synergistic effect was determined by diluting each drug to 40 mg/ml and equal volume was mixed to make up 20 µg /ml and the isolates were subjected to different concentrations of it and incubated at 37°C for 18 h. Antibacterial activity of conventional antibiotics was also tested on isolates using disc diffusion method and zones of inhibition were read according to the method of Stokes and Ridway (1980).

RESULTS AND DISCUSSION

The results show that 32 (53.3%) of the isolates were beta-lactamase producing while the remaining 28 (47.7%) were non beta-lactamase producing (Table 1). Of the 32 isolates positive for beta-lactamase, none was sensitive to methicillin, while six (21%) of the 28 isolates negative for beta-lactamase were sensitive to methicillin (Tables 2 and 3). The isolates also showed a multidrug resistant pattern to the antimicrobial agents tested except vancomycin (Tables 2 and 3). Tables 4 and 5 showed the effect of ampicillin, gentamicin and synergistic effect of both drugs on seven selected isolates and on *S. aureus* oxford strain (NCTC 6571) which was used as control.

Results from this study show a higher prevalence of beta lactamase producers than that recorded by Esumeh et al. (2004), were beta lactamase producers were 36% and non producers 63.3%. The production of beta lactamase is due to genes located on extrachromosomal plasmids. Livermore (2002) attributed this resistance to an unusual penicillin binding protein on the cell wall of such organism. The enzymes destroy the β-lactamase ring of penicillin (Gold and Moldering, 1996) and leads to an increasing resistance to the beta lactamase antibiotics.

The incidence of methicillin resistant *S. aureus* is on alarming increase and this is further confirmed in this study which showed 0% susceptilibility for beta lactamase producers and 21% for non producers. This is in line with the work done by Livermore (2000) who recorded high resistance to methicillin the very year (1961) the drug was launched. Only six (10%) of the 60 isolates examined were susceptible to methicillin and they were all non beta lactamase producers. This explains

Table 2. Antibacterial susceptibility of beta-lactamase producing stapylococci isolates no of isolates sensitive to antibacterial agents.

Isolate	Number tested	ERY	GEN	VAN	MET	AUG	TET	COT	AMX	CHL	CXC
Urine	14	1	1	9	-	3	1	-	1	2	1
Wound swab	6	-	1	5	-	-	-	-	-	-	1
Urethral swab	4	-	-	4	-	-	-	-	1	-	-
Sputum	1	-	-	1	-	-	-	-	-	-	-
Ear swab	6	-	-	5	-	1	-	-	2	-	-
Endocervical swab	1	-	-	-	-	-	1	-	-	-	-
Total %	32%	1(3%)	2(6%)	24(75%)	0(0%)	4(13%)	2(6%)	0(0%)	4(13%)	2(6%)	2(6%)

-, Resistant; COT, cotrimoxazole (25 µg); ERY, erythromycin (30 µg); VAN, vancomycin (30 µg); AMX, amoxicillin (25 µg); CHL, chloramphenicol (30 µg); GEN, gentamicin (10 µg); AUG, augumentin (10 µg); TET, tetracycline (10 µg); CXC, cloxacillin (5 µg); MET, methicillin (10 µg).

Table 3. Antimicrobial susceptibility of non b-lactamase producing isolates.

Isolate	Number tested	ERY	GEN	VAN	MET	AUG	TET	COT	AMX	CHL	CXC
Urine	6	4	4	6	1	-	-	-	1	-	-
Wound swab	6	1	-	6	1	2	1	-	2	1	1
Urethral swab	4	-	-	4	1	2	-	-	1	1	-
Sputum	3	-	-	3	1	1	-	-	-	1	-
Ear swab	3	-	-	3	-	-	-	-	-	-	-
Endocervical swab	4	-	-	4	1	-	1	-	1	-	-
Pleural aspirate	2	-	-	2	1	-	-	1	-	-	-
Total %	28%	5(18%)	4(14%)	28(47%)	6(21%)	5(18%)	2(7%)	1(4%)	5(18%)	3(11%)	1(4%).

-, Resistant; COT, cotrimoxazole (25 µg) ERY, erythromycin (30 µg); VAN, vancomycin (30 µg); AMX, amoxicillin (25 µg); CHL, chloramphenicol (30 µg); GEN, gentamicin (10 µg); AUG, augumentin (10 µg); TET, tetracycline (10 µg); CXC, cloxacillin (5 µg); MET, methicillin (10 µg).

the high resistance to the antibiotic (methicillin), which initially appeared not to be affected by the enzyme beta lactamase and thus became a salvaging agent in the treatment of many infections due to beta lactamase producing species of *Staphylococcus.* The emergence of methicillin resistant *Staphylococcus* led to the discovery of an antibiotic called vancomycin. This antibiotic has been observed to be very effective in the treatment of *Staphylococci* infections and this is proved from the results of this study. Of the 60 isolates, only seven (11.7%) were resistant to vancomycin and such vancomycin resistant *S. aureus* (VRSA) were not susceptible to any of the other antibiotics used. Thus, vancomycin could be used as a last resort in treating Staphylococcal infections. This result is in line with the work of Abbey (2004) who recorded only 15 (30%) of vancomycin resistant *S. aureus* of the 50 isolates examined. A general analysis of this study showed a trend in which there is serious antibiotic resistance from previously known antibiotics to the most recently developed ones. Also resistance could be attributed to the discovery by Sande et al. (1991) which explains that when the antimicrobial activity of a new drug is first tested, a pattern of sensitivity and resistant is usually defined. But the spectrum of activity can subsequently change because organisms have evolved the array of ingenious alterations that allow them to survive in the presence of antibiotics. The effect of ampicillin and gentamicin at different concentrations on selected isolates did not prove them to be good therapeutic agents for Staphylococcal infections. Table 4 showed that only the 20 ug/ml could inhibit the growth of Staphylococcus and this is twice the concentration in the disc. Gentamicin was a little more effective than ampicillin as the isolates showed total resistance to ampicillin. The synergistic effect did not give good susceptibility result either except for the 20 ug/ml as showed in Table 5.

This study shows a correlation coefficient ($R<1$) between beta lactamase production and multidrug resistance, between beta-lactamase production and methicillin resistance and between methicillin resistance and multidrug resistance. It was observed that beta-lactamase producing isolates have a lower susceptibility to antibiotics than non beta lactamase producers. It was

Table 4. Effects of ampicillin and gentamicin on selected isolates.

Isolate	Effects of different concentrations (µg/ml) of ampicillin and gentamicin									
	20		15		10		0.5		0.25	
	AMP	GEN	AMP	GEN	AMP	GEN	AMP	GEN	AMP	GEN
*SU (MRSA)	-	-	+	+	+	+	+	+	+	+
*SU (MRSA)	-	-	+	+	+	+	+	+	+	+
*SECS (VRSA)	-	-	+	+	+	+	+	+	+	+
“SU (MRSA)	-	-	+	+	+	+	+	+	+	+
“SW (MSSA)	-	-	+	+	+	+	+	+	+	+
“SU (MSSA)	-	-	+	+	+	+	+	+	+	+
“SP (MSSA)	-	-	+	+	+	+	+	+	+	+
NCTC 6571	-	-	+	+	+	+	+	+	+	+

SU (MRSA), Methicillin resistant *S. aureus* from urine; SECS (VRSA), vancomycin resistant *S. aureus* from endocervical swab; SWS (MSSA), Methicillin susceptile *S. aureus* from wound swab; SPA (MSSA), Methicillin susceptile *S. aureus* from pleural aspirade; +, growth; -, No growth; “ non ß-lactamase producing; ***** ß-lactamase producing; NCTC 6571, *S. aureus* oxford strain used as control.

Table 5. Synergistic effect of ampicillin and gentamicin on selected isolates.

Isolate	Effects of different concentrations (µg/ml) of ampicillin and gentamicin				
	20	15	10	0.5	0.25
*SU (MRSA)	-	-	-	+	+
*SU (MRSA)	-	-	+	+	+
*SECS (VRSA)	_	+	+	+	+
“SU (MRSA)	-	-	+	+	+
“SW (MSSA)	-	+	+	+	+
“SU (MSSA)	-	+	+	+	+
“SP (MSSA)	-	+	+	+	+
NCTC 6571	-	-	-	-	-

SU (MRSA), Methicillin resistant *S. aureus* from urine; SECS (VRSA), vancomycin resistant *S. aureus* from endocervical swab; SWS (MSSA), Methicillin susceptile *S. aureus* from wound swab; SPA (MSSA), Methicillin susceptile *S. aureus* from pleural aspirade; +, growth; -, No growth; “ non ß-lactamase producing; *****ß-lactamase producing; NCTC 6571, *S. aureus* oxford strain used as control.

also seen that all beta-lactamase producers were not susceptible to methicillin. Those resistant to methicillin had a reduced susceptibility to other antibiotics when compared to the susceptibility of the non beta lactamase producers.

From this study, it can be concluded that Staphylococci are highly resistant to antibiotics mostly penicillin because of the enzyme beta-lactamase that they produce and antibiotic resistant genes in the plasmid. However resistance can be prevented by proper hygienic practices such as proper sanitary practices by hospital staff and patients, isolation of infected patients to avoid the easy spread of antibiotic resistant species of *Staphylococcus* and optimal and judicious selection of antimicrobial agents for the therapy of Staphylococcal infection.

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Full Length Research Paper

Characterization and immobilization of partially purified alkaline protease extracted from rhizospheric soil bacterium, *Bacillus megaterium* strain EN-2 and *Bacillus subtilis* strain EN-3

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In this study, extracellular alkaline protease producing bacterial isolates EN-2 and EN-3 from the agricultural soil of C.R.C. Pantnagar were identified as *Bacillus megaterium* strains EN-2 and *Bacillus subtilis* strain EN-3 on the basis of 16S rDNA gene sequencing. During kinetic characterization, optimum pH for EN-2 and EN-3 protease activity was 10 and 9, respectively. While optimum temperature, for maximum protease activity in both isolates, was 50°C. The crude extracellular alkaline protease from isolates EN-2 and EN-3 were partially purified using ammonium sulphate fractionation and dialysis to 1.50 and 1.42 fold with 53.77% and 42% recovery respectively. The observed values of V_{max} and K_m for protease from isolate EN-2 were found to be 11.57 U/ml and 17.442 mg/ml, while for EN-3 protease these were 42 U/ml and 10.62 mg/ml, respectively. The partially purified enzyme from both bacterial strains was then immobilized in sodium alginate beads with maximum immobilization efficiency at 3% (w/w) and some change in their kinetic properties. The immobilized alkaline protease from EN-2 and EN-3 showed their maximum protease activity at pH 9 and 10, and temperature 60 and 50°C, respectively. Due to these properties, isolated extracellular alkaline proteases from the two strains are ideal choice for application in detergent formulation, leather and food industries.

Key words: *Bacillus megaterium*, *Bacillus subtilis*, alkaline protease, immobilization.

INTRODUCTION

Proteases (EC 3.2.21.24) are the single class of enzyme which occupies a pivotal position with respect to their applications in both physiological and commercial fields. These are degradative enzymes which catalyze the total hydrolysis of proteins by the cleavage of peptide bonds. Microbial alkaline proteases dominate the worldwide enzyme market, about 70% share of the detergent (Johnvesly and Naik, 2001). Among the various proteases, bacterial proteases are the most significant when compared with those of animals and plants (Gupta et al., 2002). This enzyme accounts for nearly 60% of the total worldwide enzyme sales (Adinarayana et al., 2003; Beg et al., 2003). There are acidic, alkaline and neutral proteases, but of all these, alkaline proteases are employed primarily as cleansing additives (Ward et al., 1995; Nehra et al., 2004). Alkaline protease of microbial origin possess considerable industrial potential due to their biochemical diversity and wide applications in

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tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures (Agarwal et al., 2004; Devi et al., 2008). One of the major drawbacks affecting the stability at alkaline pH of enzymes recovered from thermophiles is that enzymes from alkalophile confer stability in a wide pH range but are usually thermolabile (Griffin et al., 1992). One of the strategies to increase the enzyme stability and reusability is the immobilization. Some of the more significant advantages of the immobilized enzymes over their soluble counterparts includes enhanced stability under extreme conditions of temperature, pH and organic solvents, recovery and reuse (with their subsequent applicability in continuous processes) and in the case of proteases, removal or reduction of autodigestion (Quirega et al., 2011).

Within the different biopolymers that may be employed in such processes, Alginate is one of the most frequently used owing to that the immobilization is carried out under very mild conditions (physiological pH and temperature) without causing any deleterious alteration of the enzyme. Alginate beads have the advantages of being nontoxic, high biocompatibility and their inability to reswell in acidic environment, so acid sensitive compounds (drugs, enzymes, etc.) incorporated into the beads would be protected from gastric juice. Therefore, it is used as an entrapment matrix for cells and enzymes in pharmaceutical and food industry (Smidsrød et al., 1990).

With respect to the factors affecting culture conditions, productivity and properties of alkaline protease, purifying and characterizing this enzyme through kinetic studies by studying the effect of varying pH and temperature as the factors affecting their activity was considered significant. In the present study, we aimed to purify and immobilize extracellular alkaline protease in alginate beads from the two agriculture soil bacterial isolates screened positive for protease production and the factors affecting their activity to present potential and possible application for industrial purposes were study.

MATERIALS AND METHODS

Material, bacterial strain and growth conditions

Bovine serum albumin (BSA), and $CaCl_2$ were purchased from Sigma-Aldrich India. Sodium alginate, glycine and Tris-HCl were supplied by Qualigen. Ammonium sulphate, Luria Broth (LB) media, and Nutrient Agar media were procured from HiMedia Laboratories, India. Other chemicals of interests used were of analytical grade. Previously isolated bacterial strain in our laboratory was taken following study of Pathak (2010). The strain was cultivated in Luria Broth (LB) media.

Screening and production of proteases

Isolated bacteria from soil samples were screened for the production of protease enzyme. Skim milk agar media (Adinarayana et al., 2003) was used to screen bacteria for protease production. For the observation of protease production, bacteria were inoculated on skim milk agar plates containing 10% (w/v) skim milk, 2% agar and 0.5% NaCl. Plates were incubated at 32°C for 48 h. Clear zones of skim milk hydrolysis around the colonies gave an indication of proteolytic activity.

Protease production was carried out in a medium of the following composition: Casein 10 g, peptone 1 g, maltose 4 g, NaCl 5 g, $CaCl_2$ 0.05 g, $MgSO_4$ 0.05 g, water 1000 ml (pH 7). Cultures were incubated on a shaker (150 rpm) for 36 h at a temperature of 37°C (Venugopal and Saramma, 2006).

Partial purification of alkaline protease

Protease production was achieved by cultivation of strain in Luria Broth (LB) media. All the purification steps were performed at 4°C. Crude alkaline protease in the cell free supernatant was precipitated by adding ammonium sulphate upto 70%. The precipitates were separated by centrifugation at 10,000 rpm for 15 min and resuspended in 0.1 M phosphate buffer (pH 8) and dialyzed against the same buffer overnight with two buffer change to concentrate the enzyme.

Biochemical analysis

The proteolytic activity was determined by the modified Kunitz method using casein as a substrate (Kunitz, 1947). The supernatant of centrifuged (8000 rpm for 10 min) overnight culture broth served as the crude enzyme source. The crude enzyme (0.5 ml) was mixed with 1 ml of 2% casein dissolved in 0.1 M phosphate buffer (pH 8) and was incubated at 37°C for 30 min. The reaction was stopped by adding 3 ml of 1% trichloroacetic acid. The reaction mixture was centrifuged at 8000 g for 10 min. The absorbance of supernatant was measured at 280 nm. Amount of tyrosine produced is calculated from tyrosine standard curve. One unit of protease activity is defined as the amount of enzyme that released 1 µg tyrosine/ml/min at assay condition. All experiments were carried out in triplicate. The activity of immobilized protease was determined using procedure given above except 0.2 g immobilized enzyme taken for assay.

Bradford method was used for measuring the total protein by using bovine serum albumin (BSA) as the standard (Bradford, 1976). The protein concentration was estimated by observing the absorbance at 595 nm.

The calculation of K_m and V_{max} was done on the basis of Line-Weaver Burk Plot constructed by plotting the reciprocals of substrate (Casein) concentration on X-axis, and reciprocals of enzyme activity on Y-axis.

Enzyme immobilization

Partially purified protease in suitable amount was immobilized by dropping a 3% sodium alginate solution into a 0.25 M $CaCl_2$ solution with continuous stirring. Curing of the beads were done for 4 h in $CaCl_2$, washed several times with a 0.03 M $CaCl_2$ solution until no protease activity was observed in the final washing and stored at 4°C in the same solution prior to use. A similar method was followed for the preparation of control beads without enzyme (Betigeri and Neau, 2002).

Effect of pH and temperature on free and immobilized extracellular protease activity

The optimum pH for protease activity was studied over a pH range

of 3 to 12. Citrate buffer (pH 3-5), phosphate buffer (pH 6-8) and glycine-NaOH buffer (pH 9-12) were used to determine protease activity. The protease activity at different pH was determined. The optimum temperature for protease activity was determined by incubating the reaction mixture over the temperature range of 30-80°C at optimum pH.

Effect of pH and temperature on free and immobilized extracellular protease stability

The stability of the enzyme was determined by pre-incubating the enzyme for 1 h at 37°C with buffers of pH ranging from 3 to 12. After incubation, the residual enzyme activity (%) was measured at optimum pH. The thermal stability of the enzyme was studied by pre-incubating the enzyme at different temperature ranging from 30-80°C for 1 h at optimum pH.

Identification of bacterial isolates

Identification based on 16S rRNA gene sequence was also done. Briefly, genomic DNA from selected isolates was extracted and PCR amplification of 16S rRNA gene was carried out by using the primers: rDNA-1A (5'-AGA GTT TGA TCC TGG CTC AG-3') and RDNA-1B (5'-AAG GAG GTG ATC CAG CCG CA-3'). The PCR was done as follows: a hot-start of 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 1.5 min, and a final extension for 10 min at 72°C (Kumar et al., 2013b). Amplified PCR products were purified with QIAquick Gel Extraction kit (Qiagen, Germany) and sequenced in an automated DNA sequencer (Applied Biosystems 3730) at DNA Sequencing Facility, University of Delhi (South Campus), New Delhi, India. The sequences obtained were compared with sequences in the GenBank database from the National Centre for Biotechnology Information (NCBI) using blastn program (http://blast.ncbi.nlm.nih.gov/) and then deposited in GenBank under accession number of JN642548, and JN642549 for EN-2 and EN-3 strains, respectively. Evolutionary analyses were conducted in MEGA5 (http://www.megasoftware.net/).

RESULTS AND DISCUSSION

Screening for protease production and determination of protease activity

Bacterial strains EN-2 and EN-3 showed positive results for the production of protease by forming the zone of lysis around their colonies in skim milk agar plate. It was also confirmed by protease assay in production media in which EN-2 and EN-3 showed the protease activity (90.60 and 80.66 U/ml, respectively).

Identification of bacterial isolates

Bacterial isolates from rhizospheric soil of CRC, Pantnagar, were found to be Gram positive and rod shaped. Analysis of 16S rDNA sequence of isolates EN-2 and EN-3 with available 16S rDNA sequences in the NCBI database with 99% sequence similarity revealed identification of isolates as *Bacillus megaterium* strain EN-2 and *Bacillus subtilis* strain EN-3. The 16S rDNA sequence were submitted to NCBI GenBank database with Accession no. JN642549 for *Bacillus megaterium* strain EN-2 and Accession no. JN642548 for and *B. subtilis* strain EN-3. The phylogenetic tree of strain EN-2 and EN-3 16S rDNA sequences with 16S rDNA sequences of some protease producing isolates is given in Figure 1.

Partial purification and kinetic characterization of alkaline protease

The crude extracellular alkaline protease extracted from isolates EN-2 and EN-3 were partially purified by ammonium sulphate saturation (70%) followed by dialysis which gave 1.50 fold purification with recovery of 53.77% for EN-2 and 1.42 fold purification with recovery of 42% for EN-3 (Table 1).

On the basis of double reciprocal plot of enzyme kinetics, the value of V_{max} and K_m values for the partially purified extracellular enzyme from strain EN-2 were 11.57 U/ml and 17.442 mg/ml, respectively. In the same way, V_{max} and K_m values of EN-3 protease were 42.00 U/ml and 10.67 mg/ml, respectively (Table 2). According to the above result we can say that the isolated protease from EN-3 has more affinity for its substrate than EN-2 because of its lesser K_m value. Comparable result was reported for *B. subtilis* strain EN-4 by Kumar et al. (2013a) where the V_{max} value of extracellular protease was 22.2 U/ml.

Immobilization of protease

By varying the concentration of sodium alginate solution (1-4%), the immobilization process of alkaline protease within calcium alginate beads was carried out. High yields of immobilization were defined as the ratio of the activity of immobilized enzyme to the activity of the free enzyme used. The percentage (%) immobilization (~55% for EN-2 and 94% for EN-3) was highest at 3% sodium alginate concentration (Table 3). Leakage of enzyme occurs at lower concentration of sodium alginate due to longer pores of less tightly cross linked gel which resulted in less immobilization efficiency. An approximate 5-40% loss of enzyme activity was noticed in all cases due to denaturation of enzyme during gel formation. 3% sodium alginate concentration was found to be optimum. Although a good consolidation of the beads was obtained at 4% (w/w) sodium alginate, but a lower activity was observed. This decrease in immobilization yield with increasing sodium alginate concentration may be due to a higher density of sodium alginate at higher percentage and it did not allow the trapped enzyme to come out easily. Sodium alginate in concentration of 2-3% was used by several workers for enzyme immobilization

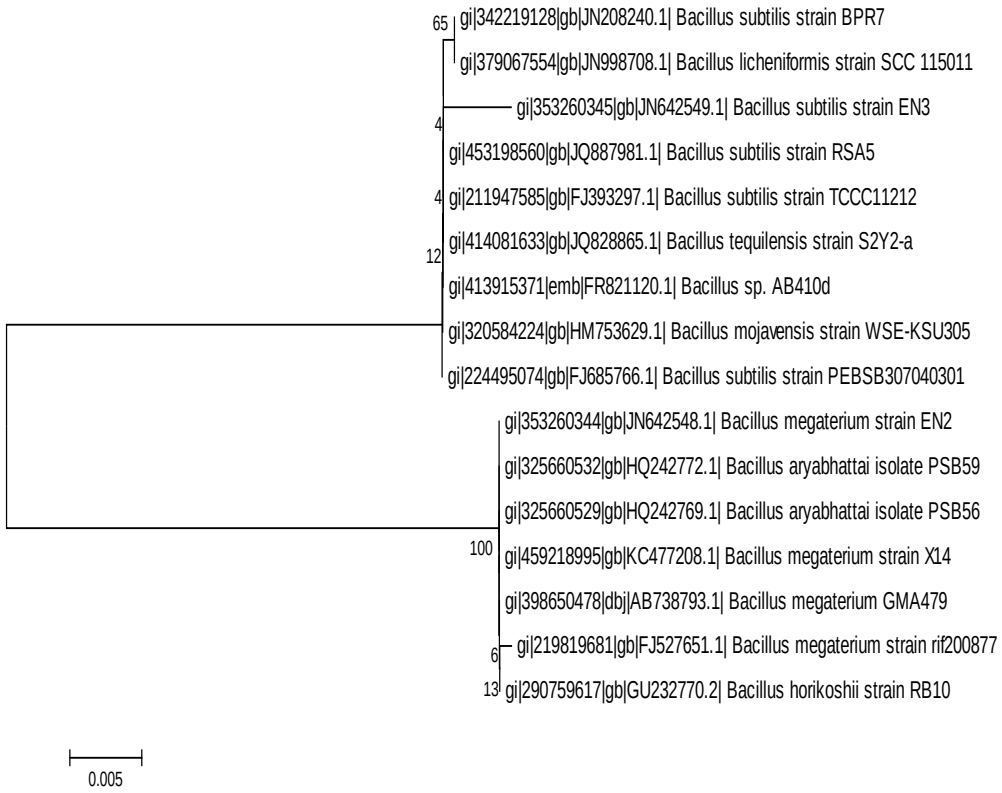

Figure 1. Phylogenetic tree of bacterial 16S rDNA sequences by neighbor-joining method.

Table 1. The purification chart of extracellular alkaline protease of EN-2 and EN-3 bacterial isolates.

Purification step	Total enzyme activity (U)	Total protein (mg)	Total specific activity (U/mg)	Fold	Yield (%)
Crude enzyme (EN-2)	106	4.1875	25.31	1	100
Fraction after dialysis (EN-2)	57	1.525	38	1.50	53.77
Crude enzyme (EN-3)	102	3.78	26.98	1	100
Fraction after dialysis (EN-3)	43.2	1.125	38.4	1.42	42

Table 2. Enzyme kinetics of the partially purified extracellular protease.

Bacterial isolates	V_{max} (U/ml)	K_m (mg/ml)
EN-2	11.57	17.442
EN-3	42.00	10.67

Table 3. Immobilization efficiency (% immobilization) of alkaline protease at different concentration of sodium alginate.

Sodium alginate	1 (%)	2 (%)	3 (%)	4 (%)
Bacterial isolate EN-2 protease	13	19.3	55	20
Bacterial isolate EN-3 protease	5	73.5	94	25

(Farag and Hassan, 2004; Mittal et al., 2005).

Effect of pH and temperature on activity and stability of free enzyme

For pH optimum, substrate solution was made in different buffers (pH 3.0-12.0), that is, citrate buffer (pH 3-5), phosphate buffer (pH 6-8) and glycine-NaOH buffer (pH 9-12). Extracellular crude protease of EN-2 and EN-3 showed its maximum activity at pH 10.0 (90.60 U/ml) and 9.0 (80.66 U/ml), respectively (Figure 2). Incubation of extracted enzyme from both strains for 60 min in different

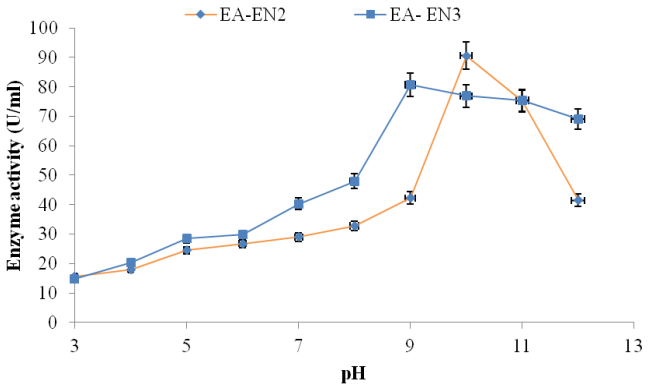

Figure 2. Effect of pH on activity of proteases from isolates EN-2 and EN-3.

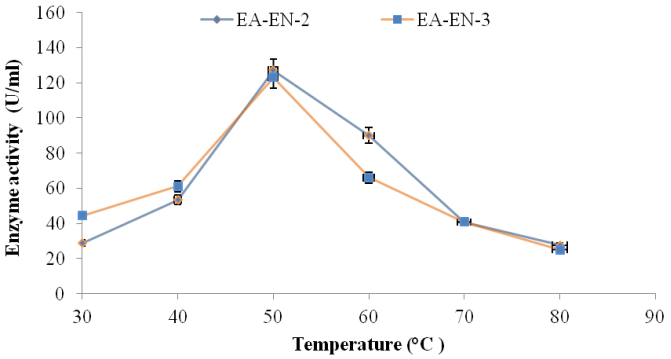

Figure 4. Effect of temperature on activities of proteases from isolates EN-2 and EN-3.

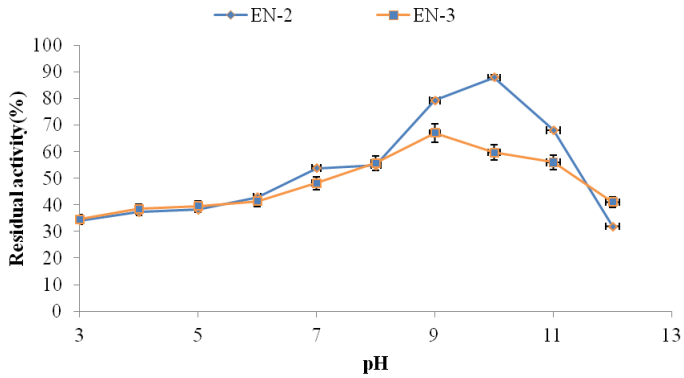

Figure 3. Effect of pH on the stability of free enzyme from isolates EN-2 and EN-3.

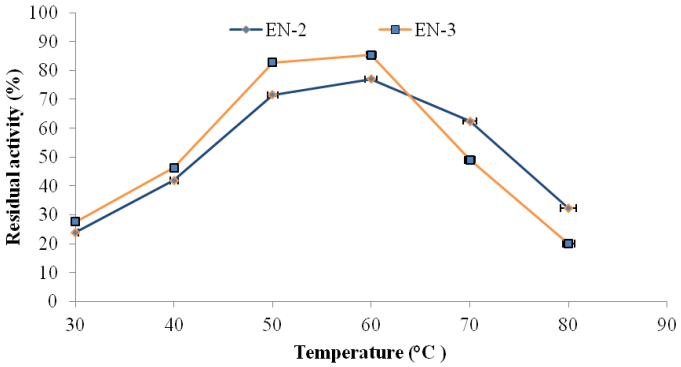

Figure 5. Effect on temperature on the stability of free protease from isolates EN-2 and EN-3.

buffers before enzyme assay indicated the stability of the enzymes from pH 8 to 11 (Figure 3). Similar results have been reported for alkaline protease from *Bacillus circulans* MTCC7906 (Jaswal and Kocher, 2007), *Bacillus clausii* (Kumar et al., 2004) and *Bacillus* sp. P-2 (Kaur et al., 2001). Thus, both EN-2 and EN-3 showed moderate protease activity at alkaline pH in cell free extract that may further be increased by varying culture conditions, gene expression and other genetic manipulations.

The effect of varying temperature on the enzyme activity was determined in the range of 30 to 80°C and optimum temperature for the maximum protease activity was found to be 50°C for both the strains (Figure 4). The isolated enzymes from both strains were stable at the broad range of temperature between 45 to 70°C (Figure 5). Thus, the isolated alkaline protease from both strains was mesophillic in nature. For *Bacillus* sp. SSR1 (Singh et al., 2001) and *Bacillus clausii* (Joo et al., 2003), the temperature for the maximum protease activity were 40 and 45°C, respectively. The results are in accordance with the study of Nascimento et al. (2004), where extracellular protease from thermophilic *Bacillus* sp. SMIA 2 was stable for 2 h at 30°C, while at 40 and 80°C, 14 and 84% of the original activities were lost, respectively.

Optimum pH and temperature for immobilized alkaline protease activity

Optimum pH and temperature for immobilized alkaline protease from isolate EN-2 were 9.0 and 60°C, respectively (Figures 6 and 7) and for the enzyme of EN-3 the pH and temperature for maximum protease activity were 10 and 50°C, respectively (Figures 6 and 7). The values of optimum temperature and pH of immobilized alkaline protease were quite different from free enzyme. This was due to some change in enzyme stability and substrate availability which was because of its immobilization.

In general, all detergent compatible enzymes should be alkaline and thermostable in nature because of high pH (8-12) and temperatures (50-70°C) in the laundry environments. Though the present protease is optimally active at 50°C, it retains more than 75% activity in the temperature range of 50-80°C, thus could have potential

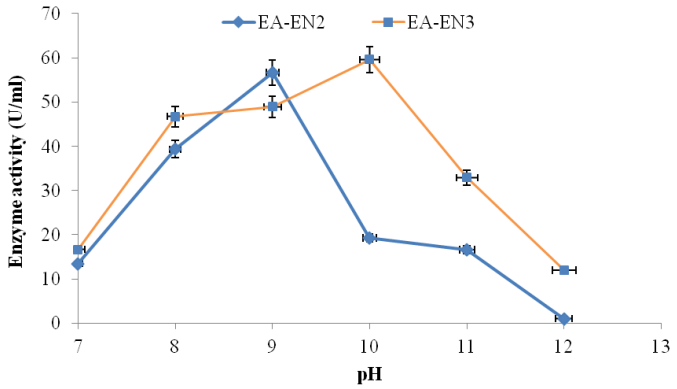

Figure 6. Effect of pH on the activity of immobilized proteases from isolates EN-2 and EN-3.

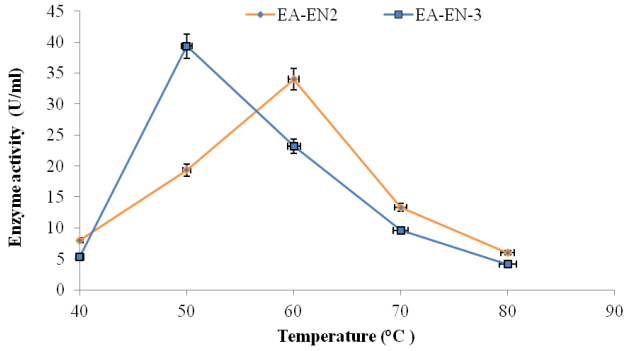

Figure 7. Effect of temperature on the activity of immobilized proteases from isolates EN-2 and EN-3.

use as detergent additives. The appreciably enzyme activity and stability at different pH and temperatures makes these isolates industrially promising and of special interests for basic and applied research.

Conclusion

In our study, proteases produced by two different *Bacillus* strains showed activity at high temperatures and pH. The alkaline proteases isolated from strains EN-2 and EN-3 are thermostable proteases. These have a relevant economical and environmental impact which alleviates the pollution problem created by leather industries as it has desirable properties such as stability at alkaline pH and high temperature.

These properties indicate the possibility of using the protease in detergent, leather and in other biotechnological applications that would require higher working temperatures. Additional work is needed for full characterization (amino acid analysis, column chromatography) of the examined proteases. For the evaluation of a biotechnological application of the protease of *Bacillus* sp., a more detailed understanding of the factors that enable this enzyme to act on compact substrates better than comparable enzymes of the same type would be helpful. Therefore, more research on the specific molecular characteristics of this interesting enzyme will be done.

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Full Length Research Paper

Isolation of two thermophilic actinobacterial strains mud volcano of the Baratang Island, India

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Studies on actinobacteria of marine habitats are gaining international importance due to proven abilities of novel compound production. Information on actinobacteria of the marine environments is less and hence the present studies were optimum at higher temperature, which indicates that they are thermophilic in nature. It can be conducted that the two strains (TA-1 and TA-2) belong to two different genera *viz. Rhodococcus* (TA-1) and *Streptosporangium* (TA-2). Actinobacterial strains were isolated from the sediment samples collected from the mud volcano, of the Baratang Island andaman and Nicobar group of islands. Out of a total of six isolates, two different actinobacterial strains (TA-1 and TA-2) were selected based on the morphological distinct colour of spore mass, riverside colour, arial and mycelia format production of diffusible pigment sporophore and colony morphology. Morphological studies indicated that the strains belonged to two different genera *viz*. *Rhodococcus* (TA-1) and *Streptosporangium* (TA-2). The strain had no aerial mycelium and analysis of the strain TA-1 showed fragmented rods, filaments and hyphae, and the strain TA-2 showed that the formed globosa sporangia on the surface of the colony liberated non-motile spores.

Key words: Thermophilic actinobacteria, isolation and identification, Baratang Island.

INTRODUCTION

Many free living microorganisms including actinobacteria have a cosmopolitan distribution and have been detected in distant habitats (Cooper et al., 2001). Of these, thermophiles represent unique and important genetic resources as their macromolecules are stable at higher temperature (Brock, 1985). Although identification of thermophilic actinobacteria continues to pose challenges, branching patterns of actinobacteria help define species (Madigan, 2006). In some species, the terminal cells in a chain turn into spores and become specialized for airborne dispersal. Heat resistance of these spores varies depending on the species (Fergus, 1967). The *thermoactinomyces vulgaris* has spores that can withstand 100ºC for 4 h. It was already shown that the thermophilic actinomycetes can produce amylases, xylanases and cellulose digesting enzymes which retain their activity at high temperatures (50-65ºC) (Kuo and Hartman, 1966; Loginova et al., 1978; Stutzenberger, 1987).

The number of thermotolerant actinomycetes in strongly heated soils of deserts and volcanic regions is comparable to or exceeds the number of mesophilic actinomycetes (Zenova et al., 2009) and their habitats and methods for isolation and recovery of thermophilic actinomycetes, such as Streptomyces, Thermomonospora and Thermoactinomyces (Edwards, 1993). Thermophilic actinomycetes were isolated from 163 (48.95%) of 333 samples of vegetable substrates and soil from different sites in Anambra and Enugu States in Nigeria (Unaogu

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et al., 1994). The thermophilic actinomycete strain 21E isolated from saline Bulgarian soils produced a highly thermostable collagenase. Macro- and micromorphological characteristics of the strain were tested on 14 media. It was concluded that the strain 21E was a typical member of the genus *Thermoactinomyces* Petrova and Vlahov (2007).

Thermophilic forms are primarily aerobic and have an optimum growth temperature range between 40 and 80°C (Tortora et al., 2007). However, a range of actinobacteria likes that of *Streptomyces thermofuscus* and *Streptomyces thermophilus* have been reported to grow at 65ºC (Waksman et al., 1939). These thermophilic actinobacteria are effective decomposers, breaking down organic matter. In India, there are only very limited studies on the thermophilic actinobacteria. Considering this, the present study on isolation and identification of thermophilic actinobacteria from the mud volcanic sediment samples of the Baratang Island, Andaman and Nicobar group of islands, was undertaken during 2007.

MATERIALS AND METHODS

Sediment samples were collected from the molten lava of the mud volcano of Baratang Island, Andaman and Nicobar group of islands. For actinobacterial analysis, pre-cleaned materials were used for collection of samples and were stored in sterilized polythene bags.

Actinobacteria were isolated from the mud volcano sediment samples adopting the spread plate technique using ISP-2 medium for isolation and enumeration of actinobacteria Sivakumar *et al,* (2005) after suitable serial dilutions. Serially diluted sediment samples were inoculated on ISP-2 medium and incubated at 55 °C for 7 days. All colonies with tough, leathery nature were counted as actinobacteria and expressed as colony forming units per gram (CFU/g). Morphologically different colonies were isolated by streaking on nutrient agar for obtaining pure cultures.

Taxonomic investigation

Growth temperature optimum

The optimal growth temperature was determined with ISP- 2 medium in the range from 25 to 75°C.

Generic level identification

Hydrolysis

Hydrolysis was made for releasing amino acids. Harvested cells of each strain weighing 20 mg were placed in an ampo bottle and 1 ml of 6N HCl was added and sealed with alcohol blast burner. The samples were kept at 121°C for 20 h in a sand bath. The bottles were cooled by keeping them at a room temperature of 28±2°C.

Thin layer chromotography

Spotting of the whole cell hydrolysates was made carefully on TLC plates using a microlitre pipette. Spots were of 5-10 mm in diameter. This was done by multiple applications on the same spot of very small portions of the sample, which were dried by hand drier (Lechevalier and Lechevalier, 1970).

Amino acids and whole-cell sugars

Each sample (3) was applied on the base lines of cellulose TLC (20 cm x 20 cm); can authentic material mixture of Diaminopimelic acid (DAP) isomes) and 1 µl of amino acetic acid (glycine) and also 3 µl sugar (Galactose, Arabinose, Xylose, Mannose) solutions were spotted as standards. TLC plate was developed with the solvent system containing methanol: pyridine: glacial acetic acid: H_2O (5:0.5:0.125:2.5v/v) and ethyl acetate: pyridine: acetic acid: distilled water (8:5:1:1.5 v/v). It took approximately more than 4 h for development. The spots were visualized by spraying with 0.4% Ninhydrin solution in water-saturated n-butanol, followed by healing at 100°C for 5 min and aniline thalate reagent (2.5 g of pthalic acid dissolved in 2 ml of aniline and made up to 100 ml with water saturated n-butanol). The spot of amino acetic acid ran faster than DAP. The sample spots were immediately compared with the spots of the standards since spots gradually disappeared in few hours and the sprayed plate was heated at 100°C for 4 min. Hexoses appeared as yellowish brown spots and pentoses, as maroon coloured spots (Lechevalier et al., 1966).

Species level identification

Aerial mass colour

The colour of the mature sporulating aerial mycelium was recorded in a simple way (White, grey, red, blue and violet). When the aerial mass colour fell between two colour series, both the colours were recorded. If the aerial mass colour of a strain to be studied showed intermediate tints, then also, both the colour series were noted. The media used were yeast Extract-Malt Extract Agar and Inorganic-Salt Starch Agar (Shirling and Gottlieb, 1996).

Melanoid pigments

The grouping was made on the production of melanoid pigments (greenish brown, brownish black or distinct brown, pigment modified by other colours) on the medium. The strains were grouped as melanoid pigments were delayed or weak, and therefore, it was not distinguishable. This is incubated as variable (V). This test was carried out on the media ISP-1 and ISP-7, as recommended by International Streptomyces Project (Shirling and Gottlieb, 1996).

Reverse side pigments

The strains were divided into two groups, according to their ability to produce characteristic pigments on the reverse side of the colony, namely, distinctive (+) and not distinctive or none(-). In case, a colour with low chroma as pale yellow ,olive or yellowish brown occurs, it was included in the latter groups (-) (Shirling and Gottlieb, 1996).

Soluble pigments

The strains were divided into two groups by their ability to produce soluble pigments other than melanin: namely, produced (+) and not produced (-). The colour was recorded (red, orange, yellow, blue, green and violet) (Shirling and Gottlieb, 1996).

Spore chain morphology

The species belonging to the genus *Streptomyces* were divided into

Table 1. Cell wall amino acids and whole cell sugars of the two strains.

Strain Number	DAP		Glycine	Whole cell sugars		Wall type
	LL-DAP	Meso-DAP		Arabinose	Galactose	
TA-1	-	+	-	-		IV
TA-2	-	+	-	-	-	III

+ denotes presence; - denotes absence.

three section (Shirling and Gottlieb, 1966), namely rectiflexibiles (RF), rectinaculiaperti (RA) and spiral (S). A drop of agar was spread well on the slide and allowed to solidify into a thin film as to facilitate direct observation under microscope. The cultures were incubated at 28 ± 2°C and examined periodically for the formation of aerial mycelium, sporophore structure and spore morphology (Shirling and Gottlieb, 1996).

Assimilation of carbon source

The ability of different actinobacterial strains in utilizing various carbon compounds as source of energy was studied following the method recommended by International Streptomyces Project (ISP) (Shirling and Gottlieb, 1966). Carbon sources for this test were arabinose, xylose, inositol, mannitol, fructose, rhamnose, sucrose and raffinose. These carbon sources were sterilized by ether sterilization without heating. For each of the carbon sources, utilization is expressed as positive (+), negative (-), or doubtful (±). In the 'doubtful ' strains, only a trace of growth slightly greater than that of the control was noticed (Shirling and Gottlieb, 1996).

RESULTS AND DISCUSSION

Population density of thermophilic actinobacteria

During the present investigation, the actinobacteria were enumerated from the sediments collected from the mud volcano samples whose population density was $2.7x10^3$ CFU/g.

Taxonomic investigation

Two different actinobacterial strains (TA-1 and TA-2) were selected based on colony morphology and detailed taxonomic investigation was carried out. Results of the analysis of cell wall components of the two strains are given in Table 1.

The strain, TA-1 possesses meso -Diaminopimelic acid (DAP) along with arabinose and galactose sugar patterns. Presence of meso-DAP, arabinose and galactose sugar patterns indicates the cell wall chemo type - IV. The strains belonging to the wall type - IV are *Rhodococcus, Microbacterium, Nocardia* and *Actinopolyspora* (Lechevalier and Lechevalier, 1970). Similarly, TA-2 possesses meso-DAP (Diaminopimelic acid) and does not contain any glycine. It had no characteristic sugar pattern. Presence of meso-DAP without any sugar pattern indicates the cell wall type - III. The strains belonging to the wall type - III are *Actinomadura, Streptosporangium, Spirillospora, Dermatophilus, Thermoactinomyces, Microbispora* and *Nocardia* Lechevalier and Lechevalier (1970).

Micro morphological character studies

The strain had no aerial mycelium. Compound microscope (400X) analysis of the strain TA-1 showed fragmented rods, filaments and hyphae. Scanning electron microscopy (15,000X) revealed that the rods are non-motile (Figure 1). The rods were non-motile and road's varied in size and shape within a single sporophore. All rods showed a smooth surface and the rods diameter varied from 0.8 to 1.2 µm and length varied from 1.3 to 2.4 µm. A gap or small plug separated some rods. These charac-ters are the typical morphological features of the genus *Rhodococcus* (Cross and Goodfellow, 1973). Based on the cell wall type and micromorphological analyses, it was confirmed that the strain TA-1 belongs to the genus Rhodococcus.

The strain had aerial mycelium. Light microscope (400X) showed that the strain TA-2 formed globosa sporangia on the surface of the colony. The fine structure of the spore was studied using light microscope (400X) and scanning electron microscopy (5,000X). It showed that the strain TA-2 formed globosa sporangia and these sporangia liberated non-motile spores (Figure 2). The spores were square in shape and non-motile. The spores contained spines in the four corners and formed short chains with not more than 10 spores in each. Spores varied in size and shape within a single sporophore. All the spores showed a smooth surface and the spore diameter varied from 0.5 to 0.9 µm and length varied from 1.1 to 1.8 µm. A gap or small plug separated some spores (Figure 2). These characters are the typical morphological features of the genus *Streptosporangium* (Couch, 1955). Based on the cell wall type and micromorphological analyses, it was confirmed that the strain TA-2 belongs to the genus *Streptosporangium*. Few workers only reported the established segmentation of both the substrate and aerial mycelia, the formation of spores with the characteristics of bacterial endospores, and the biochemical properties determined strain 21E as a representative of the genus *Thermoactinomyces* (Petrova and Vlahov, 2007).

Detailed cultural, morphological and biochemical properties of the two strains, TA-1 and TA-2 were studied and they are presented in Tables 2 to 4. The strain TA-1

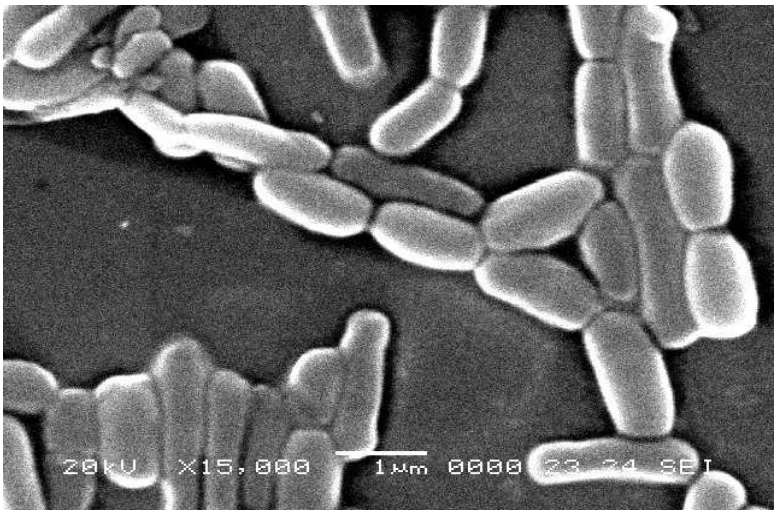

Figure 1. Scanning Electron Micrograph Non-motile rods formed by strain TA-1. (15,000X)

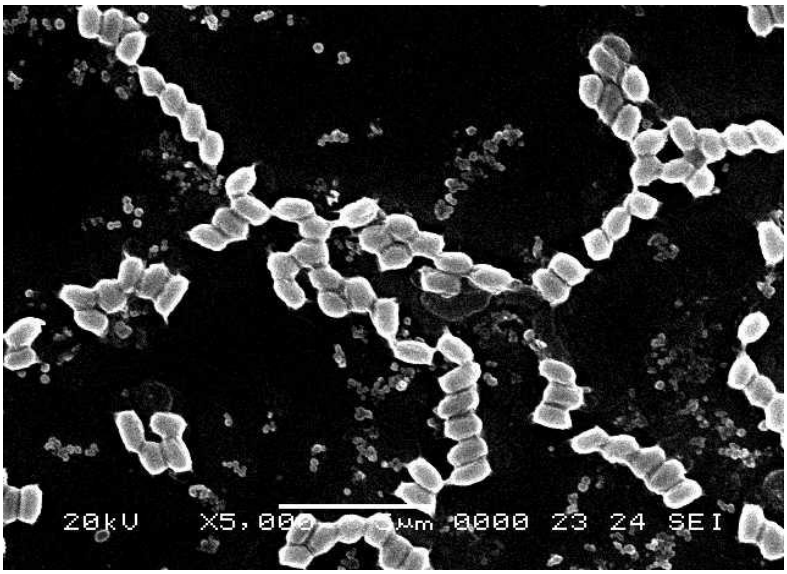

Figure 2. Scanning Electron Micrograph Non-motile spherical spores formed by the strain TA-2. (5,000X).

showed good growth on Yeast extract-malt extract agar medium after 5 days of incubation at 55°C temperatures and it showed poor growth on Starch casein agar and Glucose asparagines agarmedia. Strain TA-1 did not produced aerial mycelium but produced substrate mycelium. It did not produce any pigments such as melanoid, reverse side and soluble pigments. The spore mass of the strain TA-1 was found to be white in colour and apparent, particularly in Yeast extract-malt extract agar at 55°C temperature and 5 days incubation period. Substrate mycelia were well developed, branched and mostly unfragmented.

Cultural character studies

Like the strain TA-1, the strain TA-2 showed good growth on Yeast extract-malt extract agar medium after 5 days of incubation at 55°C and it showed poor growth on Starch casein agar and Glucose asparagines agar media. Strain TA-2 produced aerial mycelium as well as substrate mycelium. It did not produce any pigments such as melanoid, reverse side and soluble pigments. The spore mass of the strain TA-1 was found to be gray in colour and apparent, particularly in Yeast extract-malt extract agar at 55°C and 5 days of incubation period. The aerial mycelium

Table 2. Cultural and morphological characteristics of the strains TA-1 and TA-2.

Cultural and morphological characteristics	Strain TA-1	Strain TA-2
Yeast extract-malt extract agar medium	Good growth	Good growth
Starch Casein agar	Poor growth	Poor growth
Glucose asparagines agar	Poor growth	Poor growth
Aerial mycelium	Absent	Present
Substrate mycelium	Present	Present
Sporangia	Absent	present
Spore mass	White	Gray
Spore	Non-motile	Non-motile
Melanoid pigment	-	-
Reverse side pigment	-	-
Soluble pigment	-	-

Table 3. Biochemical characteristics of the strains TA-1 and TA-2.

Biochemical characters	Strain TA-1	Strain TA-2
Cellulose degradation	+	+
Hydrogen sulphide production	-	-
Nitrate reduction	+	-
Utilization of sole carbon sources		
Arabinose	+	+
Xylose	+	+
Inositol	+	+
Manitol	+	+
Fructose	+	+
Rhamnose	+	+
Sucrose	+	+
Raffinose	+	+

and substrate mycelia were well developed, branched and mostly unfragmented.

Physiological and biochemical properties

The strains TA-1 and TA-2 degraded the cellulose but they failed to produce hydrogen sulphide. The strain TA-1 reduced nitrate but strain TA-2 did not reduce nitrate. Both the strains utilized all the carbon sources *viz.* arabinose, xylose, inositol, manitol, frutose, rhamnose, sucrose and raffinose tested. The strains TA-1 and TA-2 showed good growth at 55°C and pH 7 and hence, this temperature and pH can be considered as the optimum range for these strains (Table 4). Sasagava et al. (1993) reported that the carbon metabolism is significant in taxonomy and the differences in the utilization of various carbon sources serve as additional criteria for species differentiation. International Streptomyces project (ISP) considers the utilization of nine sugars (Petrova and Vlahov, 2007).

The strain TA-1 and TA-2 showed good growth on Yeast extract -malt extract agar medium at temperature 55°C and pH 7. Below and above this temperature and pH, the strains showed poor growth. Hence, temperature 55°C and pH 7 can be detailed as, suitable for the optimum growth of the strains (TA-1 and TA-2) and these strains can be designated as thermophilic actinobacteria.

From the present study, it can be conducted that the two strains (TA-1 and TA-2) belong to two different genera *viz. Rhodococcus* (TA-1) and *Streptosporangium* (TA-2) whose growth was optimum at higher temperature which indicates that they are thermophilic in nature. There are few works on thermophilic actinobacteria from Aarthi (2007) who isolated actinobacterial strains from the mud volcanic samples and identified there as *Rhodococcus* like organisms which required higher temperature (50-60°C) and also reported the temperature range between 35 and 65°C, and did not grow at 33 and 67°C. The optimal growth temperature was from 55 to 60°C. Good growth occurred in the presence of 3-7% and it was weak at 10% NaCl (Petrova and Vlahov, 2007).

Further studies are needed to identify these two strains (TA-1 and TA-2) up to the species level through the conventional as well as molecular methods. Studies are also required to test the potential of the two strains (*Rhodococcus*

Table 4. Physiological characteristics of the strains TA-1 and TA-2.

Physiological characteristic	Strain-TA-1	Strain-TA2
Temperature range (°C)		
35	Poor growth	Poor growth
40	Poor growth	Poor growth
45	Moderate growth	Moderate growth
50	Good growth	Good growth
55	Good growth	Good growth
60	Good growth	Good growth
65	Moderate growth	Moderate growth
pH range		
5	No growth	No growth
6	Poor growth	Poor growth
7	Good growth	Good growth
8	Moderate growth	Moderate growth
9	Poor growth	Poor growth
10	No growth	No growth

and *Streptosporangium*) for the production of enzymes which could be stable at higher temperatures, as *Rhodococcus* and *Streptosporangium* were isolated from the mud volcanic samples where the minimum temperature was 50°C. If thermostable enzymes could be derived from these two strains, then they may be explored for this potential application in industries using enzymes at higher temperatures.

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Full Length Research Paper

Sodium-potassium ionic ratio correlates with yeast induction from *Mucor circinelloides* Tieghem

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A critical feature of fungal dimorphism is the morphogenetic conversion from mold to the yeast form. In the conversion of a fungus to the yeast form, little is known about the morphological changes that occur prior to the latter. A previous study from our laboratory showed that K^+ plays important role in the generation of protoplasts from sporangiospores; other morphologies exhibited included thallo-arthric-, holothallic-, holoblastic conidia as well as septate hyphae with provenant vesicular chains of conidia and yeast cells but phenotypic variabilithy of stable forms also exhibited in synthetic broth in a study conducted at pH 4.5 and temperature 20°C. This was confirmed in this study. We further show that phenotypic modification resulting in several transient forms occurred during the early growth phase that led to conversion of germ cell to neoplast, then through protoplast to prevegetative cell and nascent yeast. On modulating K^+ concentrations with Na^+, growth pattern exhibited was either biphasic or sigmoid, which was at optimal at yeast induction sigmoid curve and its phases correlated with dynamic changes in ionic flux, with a Na^+/K^+ ratio of 0.78 at lag- and 2.90 at exponential phase. It was thought that in the pH - profile two-phase anisotropic growth environment, transmembrane proton ion gradient favoured ionic circulation that triggered phenotypic modification in which protoplasts were generated and, subsequently, prevegetative cells evolved from simultaneous with metabolic adjustment. Subsequently, cells became vegetative in the yeast form. Our data suggest that diminishing logarithmic growth in the sigmoid pattern was triggered by rapid Na^+ extrusion from intracellular medium of the induced yeast.

Key words: Sporangiospore, synthetic broth, yeast cells, proton gradient, Na^+/K^+ ratio.

INTRODUCTION

The phenomenon of fungal dimorphism has been a subject of investigation not only because it serves as a model for morphogenesis in the life sciences but has heightened because many dimorphic species exist that are clinically and agriculturally important, as they become invasive in one expressive growth habit or the other. Structural forms that are subject to conversion include hyphae, conidia, spores, germ tube, pseudohyphae, spherule and yeast cells.

The precise morphological form taken, filamentous or unicellular, has been attributed to environmental factors. Although elevated temperature is trigger for morphogenetic switching of many pathogenic fungi, including *Histoplasma capsulatum, Coccidioides immitis*, *Paracoccidioides brasiliensis, Penicillium marneffei, Sporothrix schenckii*, factors like pH (Dede and

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Okungbowa, 2009), or transition metal ions (Yamaguchi,1975) contribute to *Candida albicans* dimorphism. In the case of *Ustilago maydis*, sexual elements are involved as fusion of compatible unicellular forms switch to invasive dikaryotic hyphal phase sustaining growth as such in the presence of the host plant (Bolker, 2001).

Although, for another agriculturally important pathogen, *Ceratocystis ulmi*, a nutritional factor proline triggered the dimorphic switching to yeast form (Kulkarni and Nickerson, 1981). The ability to undergo morphogenetic transition was thought to be essential for pathogenesis. Since the concern here is the development of a disease, the chain of events leading to establishment of a stable morphology, filamentous or unicellular, is essential to the development of the disease.

We have used *Mucor circinelloides* in our investigations on the dimorphic switching because its growth expression has proved to be multifaceted: thallo-arthric-, holothallic-, holoblastic-, septate filamentous with vesicular concurrent catenate conidia and terminal budding yeast cells (Omoifo, 2012; Omoifo and Awalemhen, 2012); this is apart from the multipolar budding yeast-like morpho-logy that induced under 30% CO_2 pressure (Bartnicki-Garcia and Nickerson, 1962; Lubberhusen et al., 2003; McIntyre et al., 2002) or phenetyl alcohol treatment (Terenzi and Storck, 1968) as well as the normal aerobic coenocytic filamentous growth form. These asexual structures produced by *M. circinelloides* were proliferating but unicellular forms produced non-persistent buds.

Prying into the switching to unicellular growth habit becomes an intriguing exercise as preliminary electrophysiological study indicated that proton flux across the biomembrane of a growing cell in a biphasic anisotropic growth environment permitted the morphogenetic conversion (Omoifo, 2012). Within such milieu, ionic circulation occurred between the bulk and intracellular media (Omoifo and Awalemhen, 2012). Thus, Na^+ and K^+ antiport transport mechanism ensued on each side of the divide and this coincided with the transient cryptic forms that accretion ally yielded at optimum conditions, the stable but non-persistent unicellular morphology, which is terminal budding yeast cell.

In this transformation process, we observed a directional assembly of morphogenetic sequences; such cryptic demarcations were modeled as such: sporangiospore-germ sphere-neoplasm-neoplasts-protoplasts-nascent yeasts-terminal budding yeasts (Omoifo, 2009). There is the need to understand the nature of these discontinuities and how they are associated with Na^+ and K^+ flux through the biomembrane of the growing microorganism in this aforsaid biphasic anisotropic environment.

In this study, we confirm that K^+ is absolutely necessary for the generation of protoplasts in phase 1 and, also Na^+ accumulation played a role in yeast form evolvement in phase 2 of the pH profile when there was correspondingly enhanced level proton-release intensity followed by a diminishing level, attributable to activities of the growing microorganism, but fundamentally, following the establishment of transmembrane proton ion gradient, the Na^+/K^+ ratio was more critical in the induction of yeast cells from sporangiospores. A deviation from the optimal ratio for yeast cell induction possibly led to differential expression of other morphological components including the thallic subtypes initiated at the germ sphere composite.

MATERIALS AND METHODS

Fungal strain and maintenance

The organism, *M. circinelloides* Tieghem, used in this study was first isolated from decayed fruit of soursop, Annona muricata L., obtained from the floor bed of the tree. It has been used in earlier studies (Omoifo, 2006a; b, 2012; Omoifo and Awalemhen, 2012). It was maintained as glucose- yeast extract- peptone (GYP: 10: 03: 5 g/l) solid cultures where it exhibited filamentous growth habit. A fresh culture was prepared after seven days.

Inoculum preparation for growth studies

Inoculum was obtained by pouring deionized sterile distilled water over aerobic growth and a sterile glass rod gently passed over the surface so as to dislodge the spores. The suspension was poured into centrifuge tubes and spores washed by centrifuging at 5000 rpm for 7 min at 25°C in an MSE 18 centrifuge. The wash was decanted, sediment re-suspended and further washed with two changes of sterile distilled water. Spore count was taken with Neubauer haemocytometer (BSS No. 784, Hawksley, London vol. 1/4000) and the suspension was adjusted to 1 million spores per ml in deionized sterile distilled water, using a tally counter in the quantification of spores with the aid of a tally counter.

Reagents and culture media

All reagents for the culture medium were obtained from BDH Laboratory supplies (Poole, UK). Media were prepared per litre of glucose, 10.0 g; (NH4)2SO4, 5.0 g ; $MgSO_4.2H_2O$, 2.0 g; $FeSO_4.7H_2O$, 0.10 g; MnCl2, 0.065 g; $CuSO_4.7H_2O$, 0.06 g; $ZnSO_4.5H_2O$, 0.06 g; NaCl, 0.10 g. Media were prepared in 5000 ml beakers. Weights of buffer components (0.2 M Na2HPO4, 0.1 M) citrate were obtained using H54AR mettler balance and added to the beaker.

In the first set of experiments, the effect of K+ was tested; each of duplicate flasks was incorporated with the various concentrations of KH_2PO_4: 0.0, 0.50, 0.70, 0.90, 1.00, and 1.10 g/l. In the second set of experiments, three levels of K+ which were found outstanding to yeast induction were chosen and varied with four levels of Na+. Therefore, to test the effects of K+ and Na+, each of duplicate flasks was incorporated with the various concentrations of KH2PO4: 0.90, 1.00, and 1.10 and of NaCl: 0.05, 0.10, 0.15, and 0.20 g/l. The pH was adjusted to 4.5 with 2 N NaOH or 1 N HCl, using a Cole-Parmer pH Tester model 59000, in the 5000 ml beakers before dispensing in an 80 ml of broth in each of duplicate 250 ml Erlenmeyer flasks for each test. The solution in each flask was made up to 100 ml with glass distilled deionized water and sterilized at 121°C for 15 min.

Table 1. K^+ supplementation, biomass estimates with their respective standard errors and form of growth during the cultivation of *M. circinelloides* in synthetic broth for 120 h at pH 4.5 and 20°C, ambient temperature.

Treatment	Mean biomass and standard error at 520 nm	Form of growth
K^+- level (g/l)		
Control, 0.0	0.706 ± 0.043	Hc, Ec, Ht, Ms, Gs.
0.50	1.155 ± 0.246	Y, N, Hc, Ec, Ht, Ms
0.70	0.744 ± 0.243	Y, Hc, Ec, Ht, Ms
0.90	0.876 ± 0.210	Y, Mf, P, Hc, Ec, Ht,
1.0	0.896 ± 0.052	Y, Hc, P, Mf
1.10	0.858 ± 0.060	Y, Mf, P, Hc, Ec, Ht,

Ec, *Thallic conidia*; Hc, holoblastic conidia; Ht, holothallic conidia; Mf, mycelia fragment; Ms, septate mycelia fragments, Gs, growth sphere; N, granular particles/neoplasts, protoplasts.

Table 2. Homogeneous subsets of mean biomass estimates obtained during sporangiospore-yeast transformation of *M. circinelloides* cultivated in K^+- incorporated synthetic broth for 120 h at pH 4.5 and ambient temperature of 20°.

Treatment (K^+ g/l)	Mean
Subset 1	
0.50 K^+	1.1550
1.00 K^+	0.896
Subset 2	
Control	0.706
0.70 K^+	0.744
0.90 K^+	0.876
1.10 K^+	0.858

Means were separated using l.s.d., $p < 0.05$, 0.2691. No yeast cells were observed in the control treatment; although 0.5 g/l treatment had the highest biomass, there was a preponderance of thallic subtypes, including determinate thallic conidia, holothallic conidia, holoblastic conidia; yeast cells constituted the greater propotion of morphologies in the 1.0 g/l K^+ treatment.

Inoculation, growth conditions and sample collection.

A 1 ml of spore suspension was drawn and inoculated into each broth flask using a 0.5 ml rubber suctioned pipette in a laminar flow chamber, model CRC, HB-60-180. The inoculum flask was shaken at each operation so as to keep the spores in suspension. Each culture flask was then shaken for 30 s and thereafter incubated at 20°C in a preset cooled Gallenkamp incubator. At 24 h interval the culture flasks were brought to the inoculating chamber. The flasks were shaken; 10 ml of broth was withdrawn and deposited into factory- sterilized plastic sample tubes, pre-labeled for each experiment. The culture flasks were returned for further incubation and samples kept at -18°C until analysis.

Biomass determination

Culture broth samples were thawed up to room temperature before biomass determination. This was done by measurement of optical density at 520 nm. This wavelength was chosen because ordinarily $FeSO_4$ impacted greenish coloration on culture broth. Absorbance was determined with a Grating spectrophotometer CE 303 (Cecil Instruments, Cambridge).

Cellular concentration of cations

For determination of intracellular ion content, the method followed was that of Camacho et al. (1981) with modifications. The culture suspensions were centrifuged at 5000 rpm for 10 min, at 25°C (MSE 18). The supernatant was each decanted and 5 ml of 20 mM $MgCl_2$ solution added and centrifuged for 10 min, in a very rapid operation. A 5 ml of 0.2 M HCl and 5 ml of 10 mM $MgCl_2$ were added to re-suspend the cells. These were then poured into factory-made sterile plastic bottles thereafter left for ion extraction for 24 h. The extract was centrifuged for 15 min and the supernatant was obtained for cation determination using Digital Flame Analyzer (ref. FGA -350-L; Gallenkamp, England).

Statistics

Results were subjected to analysis of variance (ANOVA) test for a split-plot format for combined factors and considered significant if $p < 0.05$ and comparison between means was performed using the Genstat 5 package.

RESULTS

Effect of K^+ on biomass

Growth of *M. circinelloides* occurred at all K^+ levels tested. The experimental values for mean biomass, as optical density, for the cultures with sporangiospores as inoculums, are shown in Table 1. Mean biomass did not increase as the concentration of K^+. Variability in growth in the K^+ supplemented batch broths was greater than that in the control study as reflected in the corresponding standard errors (Table 1).

A 2- way analysis of variance showed that K^+ had significant effect on biomass production at $p<0.05$. But time and, the interaction of time with K^+- level did not (Supplementary Table 1). Sifting of means in order to know the contributors to the difference at this level (L.S.D. at $p<0.05$, 0.2691) gave two homogeneous subsets (Table 2). The least value, which was obtained in

Table 3. Homogeneous subsets of mean biomass estimates obtained during sporangiospore-yeast transformation of *M. circinelloides* cultivated in K^+- and Na^+ incorporated synthetic broth.

Treatment (K^+g/l)	Mean
Subset 1	
0.10 g/l Na^+ (g/l)	0.3774
Subset 2	
0.50 g/l Na^+	0.3735
0.15 g/l Na^+	0.3320
0.20 g/l Na^+	0.3121

Means were separated using L.S.D., $p < 0.05$, 0.0494. Note: protoplast or yeast cell was not observed in the control treatment in table 3, that is, multiionic broth without K^+; therefore, it was unneccessary to include the control in this subtest as it was the effect of Na^+ modulation on K^+ effect, that is, protoplast generation, that was being tested here.

the control study, shared a similarity with 0.70, 0.90 and 1.10 g/l K^+. The second subset that contained the highest biomass value had two concentration levels with similar significance; 0.50 and 1.0 g/l K^+.

In a biotechnological process, assumption of regular growth pattern by a microorganism lends itself to determination of parameters like growth rate, specific product concentration necessary in order to know the basic route to increasing productivity and or improving physiological relationship. The lag phase, exponential phase and stationary phase are well known underlying orientation for microbial growth. Biomass profiles (not shown) however were a- typical of sigmoidal growth pattern at all the K^+ - levels tested.

Effect of K^+ on morphological expression

Microscopic examination showed that variability was highly reflected in the morphologies induced. Thallic growth, septate, and did not form meshwork, occurred in different subtypes, including conidiophore terminating in conidiogenous vesicle with concurrent catenate conidia, determinate thallic conidia, holothallic conidia and holoblastic conidia. Similar morphologies also occurred at 0.50 g/l K^+, but a striking difference was the occurrence of granular/neoplastic units and emergent/nascent yeasts unbudded), although scanty at this K^+- level. At 0.70 g/l K^+ supplementation, neoplasts which were more preponderant became larger in sizes in contrast to the preceding concentration. Few terminal budding yeast cells, which assumed various shapes and sizes, were observed. Preponderance of thallic subtypes as observed in the control broth diminished still in the 0.90 g/l K^+ supplemented broth while neoplasts became more numerous and larger in sizes; they assumed a tender membrane and were globose or rod shaped. These were the protoplasts. Also there were a few enlarged yeast cells. At 1.0 g/l K^+-level, thallic presence became scantier. Protoplasts assumed internal dimensions and were very robust. Although there were few nascent yeast types, enlarged and matured terminal budding yeast cells were preponderant. At the highest K^+ supplemented broth, protoplasts were globose to subglobose in shape and were not very numerous; nascent/emergent yeast cells were rods, subglobose, ovoidal, obpyriform to ellipsoids. Other morphologies observed were germlines, septate thallic growth with vesicular conidial head groups, holoblastic- and holothallic conidia, which became more robust.

Effect of combined ionic incorporation on morphological expression

Like the single factor K^+ supplemented broth in experiment 1 above, reproductive structures formed in the Na^+ and K^+ modulated broths included the various thallic subtypes. But the predominant morphology was terminal budding yeast cells, observation of which showed to be of higher magnitude than what occurred in the single factor K^+ supplemented broths. This perhaps indicated an additional effect as a result of Na^+ modulation.

Effect of combined ionic incorporation on yeast induction

A 3- factor analysis of variance indicated the significance of individual or combined effects of the elements on growth. Results (Supplementary Table 2/Table 4) show that the main factors Na^+, $p<0.05$, and time, $p<0.001$, contributed significantly to growth, but K^+ alone or the 3, 2-way interactions and 1, 3-way interaction combining K^+, Na^+ and time did not. A separation of means was done using the l. s. d. 0.04944, $p<0.05$, to locate what level of Na^+ contributed to the significance. This resulted in two homogeneous subsets, with 0.10 g/l Na^+ level making a distinct subset.

This is shown in Table 3. Histogrammatic represen-tation further confirms this (Figure 1a). It also shows that beyond this level of Na^+ incorporation, biomass was suppressed. What this suggested is that it was the incorporation of Na^+ that further enhanced or, added value to the induction and proliferation of yeast cells in the combined factor broths but in a time dependent manner.

When plots of interaction of the significant factors with the other elements were thus obtained (Figure 1), patterns generally exhibited 2-phase growth. At the 0.9 g/l K^+ treatment, O.D. did not differ from 1.0 g/l K^+ supplemen-

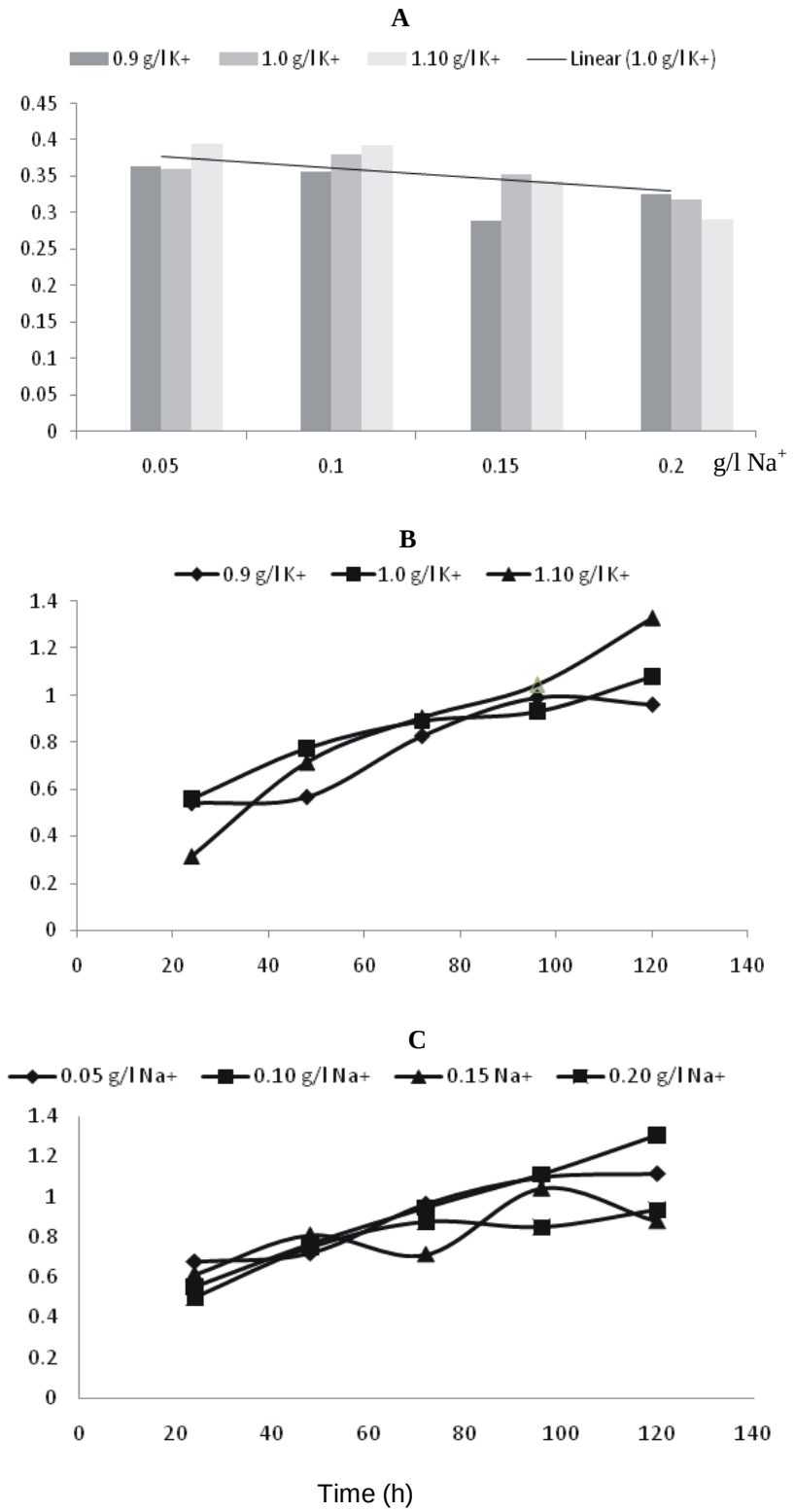

Figure 1. Effect of K^+ and Na^+ supplementation on biomass generation of *M. circinelloides* cultlivated in multiionic broth. A, extracellular K^+-time profile; B, extracellular Na^+-time profile; C, K^+ & Na^+ simultaneous supplemention of broth. Although there were multiple phenotypic expression, observation showed that protoplasts and yeast cells predominated at 1.0 g/l K^+: 0.1 g/l Na^+.

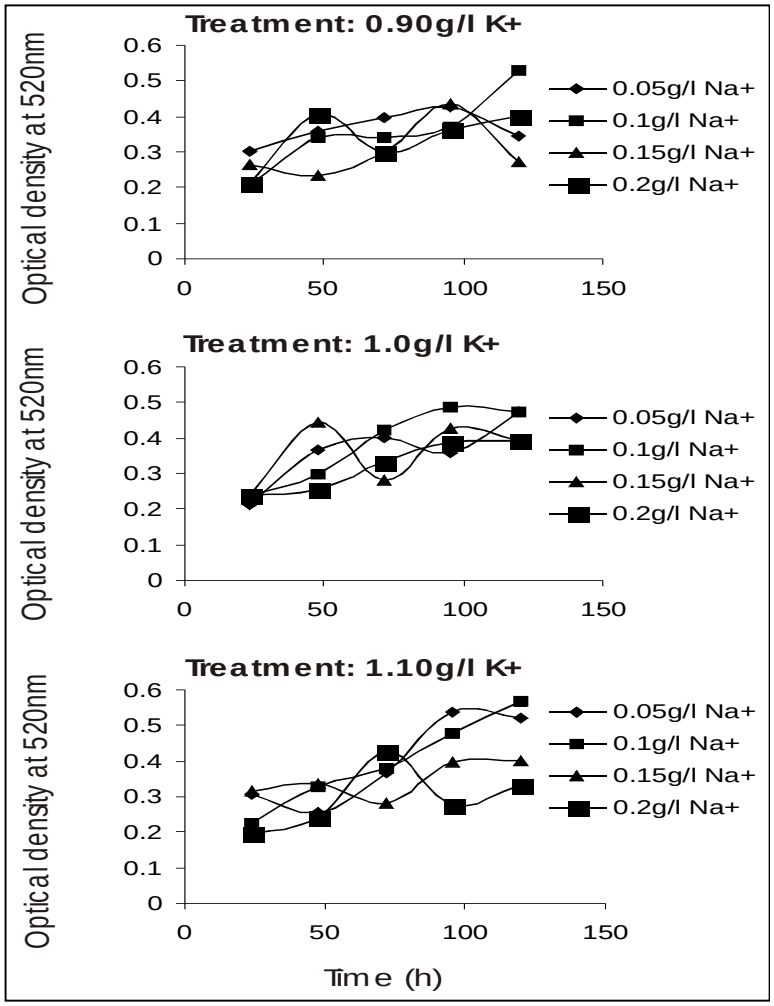

Figure 2. Biomass profiles of *M. circinelloides* cultivated in K^+ incorporated synthetic broth with Na^+ supplementations.

tation when modulation was with 0.05 g/l Na^+, but it was lower at 0.10 and 0.15 g/l Na^+ and thereafter upshot the latter treatment at 0.20 g/l Na^+ (Figure 1a). At the higher level treatment (1.10 g/l K^+), 0.05 g/l Na^+ modulation stimulated higher growth in comparison with the same level when K^+ supplementation was with 0.9, or 1.0 g/l K^+; growth was however at par with 1.0 g/l K^+ supplementation when modulation was with 0.10 g/l Na^+. Growth subsequently diminished to a lower value than that at 0.9 or 1.0 g/l K^+ treatment.

What this indicate is that at 1.0 g/l K^+-level, broth modulation higher than 0.10 g/l Na^+ suppressed biomass accumulation. The fluctuation in growth on modulation of the 0.9 g/l K^+ broths with Na^+ made this treatment unreliable for further conduct of our study on fungal dimorphic switching.

Therefore, 0.1 g/l Na^+ modulation became the optimum for biomass production with growth value lower at either side of this level.

Effect of significant factor incorporation on growth pattern

A 2- phase growth pattern was very conspicuous at time versus K^+: 0.9, 1.0 g/l (Figure 1b) and time versus Na^+: 0.15, 0.20 g/l (Figure 1c) interaction. However, subtle sigmoid pattern was exhibited at time versus K^+: 1.10 g/l (Figure 1b) interaction. Variation between these two growth habits occurred at the other levels.

Na^+ modulation and growth profile

We decided to look at growth profiles at specific K^+ - levels with the various Na^+ modulation. Although only one

peak occurred at 0.05 g/l Na^+-and 0.15 g/l Na^+, growth patterns were not sigmoid (Figure 2a). Growth at the other Na^+-levels were bi-phasic where the 2nd peak was still rising at termination of experiment (Figure 2a). The bi-phasic pattern was very conspicuous at time versus K^+: 1.0 with 0.15 g/l Na^+ modulation, while at 0.05 g/l Na^+ the 2nd peak was still rising at termination of experiment. However, striking sigmoid pattern debuted at 0.10 g/l Na^+ modulation, and less strikingly so at 0.20 g/l Na^+ incorporation (Figure 2b).

At 1.10 g/l K^+ treatment, approximation of bi-phasic patterns were also observed at 0.15 Na^+ and 0.20 Na^+ incorporation, although the 1st peak at 0.20 g/l Na^+ occurred where there was a median trough for the 0.15 g/l Na^+ profile.

At 0.10 g/l Na^+, modulation growth was still rising at termination of experiment, while profile at 0.05 g/l Na^+ was a deviant sigmoid, where at lag phase there was an initial fall in growth before subsequent apparent exponential growth (Figure 2c). Variation between these two growth habits occurred at the other levels.

Two main patterns were pronounced when biomass profiles were obtained for the Na^+ versus K^+ interactions (Figure 2a-c). Near- perfect sigmoid curves were obtained at 1.0 g/l K^+ versus 0.10 g/l Na^+ and 1.0 g/l K^+ versus 0.20 g/l Na^+ (Figure 2b) while near-perfect bi-phasic growth profile exhibited at 1.0 g/l K^+ versus 0.15 g/l Na^+ (Figure 2b) and 1.10 g/l K^+ versus 0.15 g/l Na^+ (Figure 2c). Exhibited growth patterns at the other levels were variations of these two identified patterns, sigmoid and bi-phasic.

Regression analysis of optical density on external and internal ionic composition

The observation on the two standard patterns led us to regress the principal variate, optical density, on the major external and internal ionic composition which had determinable values including K^+_{ext}, Na^+_{ext}, K^+_{int}, Na^+_{int} at $p<0.05$. This was done in order to partition the contribution of the factors to biomass build-up, and possibly form development. Results (Supplementary Table 3) show that these factors apparently did not make significant contribution at $p<0.05$ as only 1.9% of the variability within was accounted for (std error, 0.186).

Yet ionic circulation was inherent during the growth of the microorganism. This shows that the analysis of variance was unable to account for the behaviour of the microorganism in our minimal medium.

Transmembrane proton gradient and ionic flux

Thereafter, we looked at the differential of pH of bulk medium from the initial value of 4.5; profiling clearly showed the same regular bi-phasic pattern, no matter the exhibited growth habit, which was either bi-phasic or sigsigmoid. Figure 3 illustrates this. There was initial decrease of bulk medium pH, possibly indicating H^+ extrusion from intracellular medium and a subsequent reversal after the point of inflection, also showing a possibility of indicating H^+ uptake by each cellular unit. This could mean a change in direction of transport process for H^+ through the biomembrane of the growing microorganism. Thus, in each phase, the medium was anisotropic, possibly permiting transmembrane proton gradient.

If we look at the treatment, 1.0 g/l K^+: 0.10 g/l Na^+ where near-perfect sigmoid growth habit exhibited, ionic communication involving particularly H^+, Na^+ and K^+ occurred between the external and intracellular media. For Na^+ and K^+, Figure 4 illustrates this. At the lag phase, there was simultaneous influx of K^+ and efflux of Na^+. A reversal of this occurred after 48 h of growth. This was followed by very rapid influx of Na^+ up till mid log phase where K^+ efflux was slow, but became dramatically steeply, correspondingly with efflux of Na^+ from mid log phase till 96 h after inoculation. There from, influx of both K^+ and Na^+ occurred at the stationary phase of growth.

Intracellular ionic accumulation, growth habit and morphological expression

Histogram of ionic data generated was juxtaposed with that of biomass and pH differential. This showed that the H^+ - release intensity was highest at 72 h after inoculation (Figure 5a). This was at mid log phase when there was also the highest intracellular accumulation of Na^+ (Figure 5b). Such H^+ - release stabilized until biomass peaked at 96 h after inoculation (Figure 5a), but by this time intracellular Na^+ content diminished; although there was a rise in this level at stationary phase, it was not half as much as that at mid log phase. On the other hand, the H^+ level diminished at the stationary phase. Using this as standard, Figure 6 shows mid log phase accumulations of Na^+ in the various treatments and the growth habit thereupon exhibited. At 0.90 g/l K^+ level, maximal Na^+ accumulation occurred at 0.05 g/l Na^+ modulation. This decreased with increase in Na^+ incorporation. Growth pattern at 0.05 g/l Na^+ broth was single phase, peaking at 96 h after inoculation. This treatment also induced the least proportion of yeast cells, but proportion of yeast cells increased with rise in Na^+ incorporations as growth pattern became bi-phasic.

In comparison with the 0.9 g/l K^+ level, yeast cells were more preponderant in treatments with 1.0 g/l K^+ supplementation. There were bi-phasic growth expressions in media incorporated with 0.05 and 0.15 g/l. Na^+ and intracellular Na^+ accumulations respectively were 8.40 and 11.0 mg/kg, which were much lower than intracellular accumulations at 0.10 g/l Na^+ modulation that promoted sigmoid growth habit and induced predominantly yeast

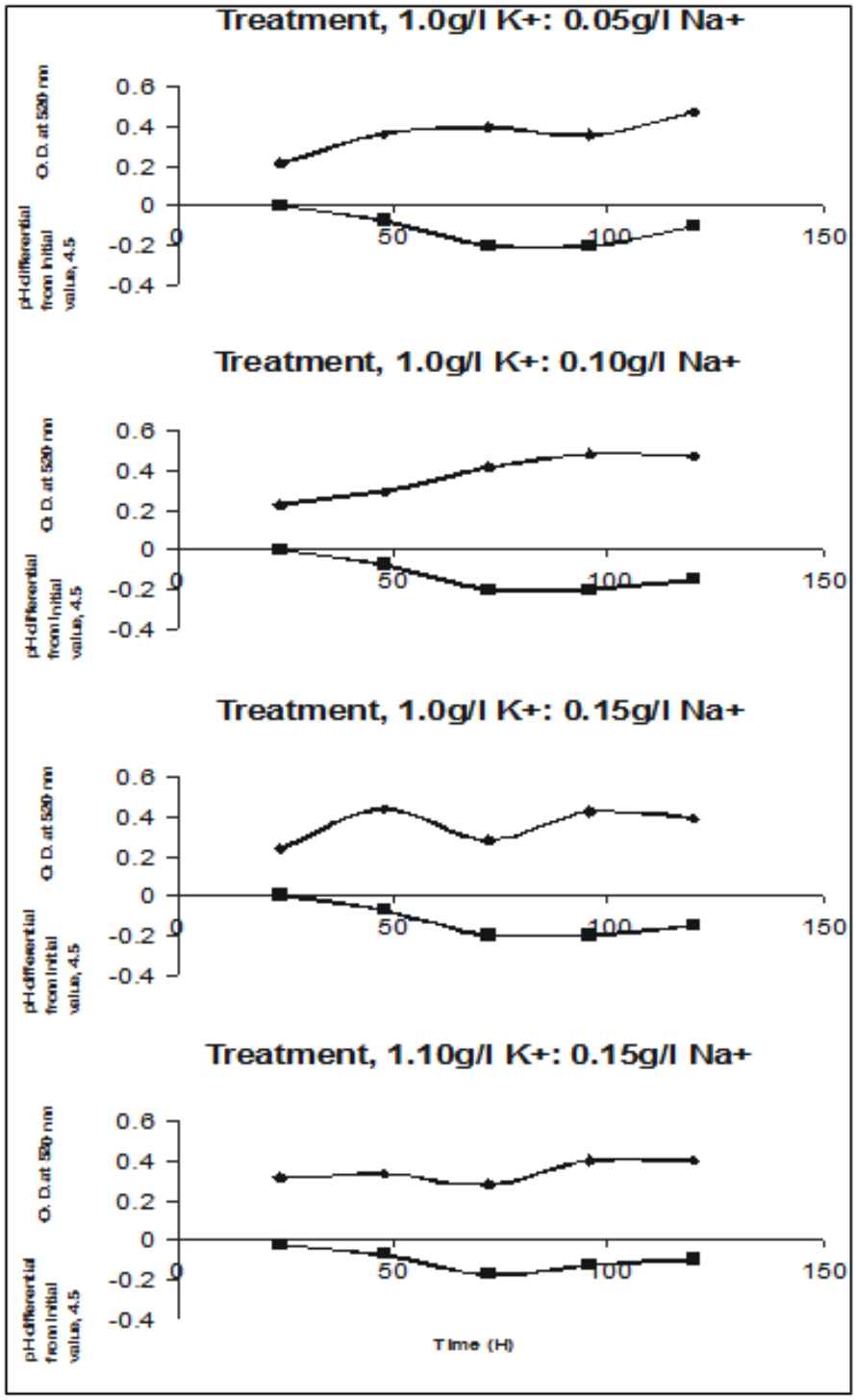

Figure 3. Treatments showing representative growth patterns, sigmoid and two- phase habit, and pH profiles during the cultivation of *M. circinelloides* in synthetic broth. Note: whatever the growth habit, the pH profile showed similar pattern.

cells. Sigmoid pattern was also exhibited at 0.20 g/l Na^+ level, although Na^+ accumulation was minimal.

Profiling of growth in broths with 1.10 g/l K^+ levels gave bi-phasic growth patterns, except with 0.10 g/l Na^+ incorporation where growth was apparently exponentially determined. At the 1.10 g/l K^+ treatment, intracellular Na^+

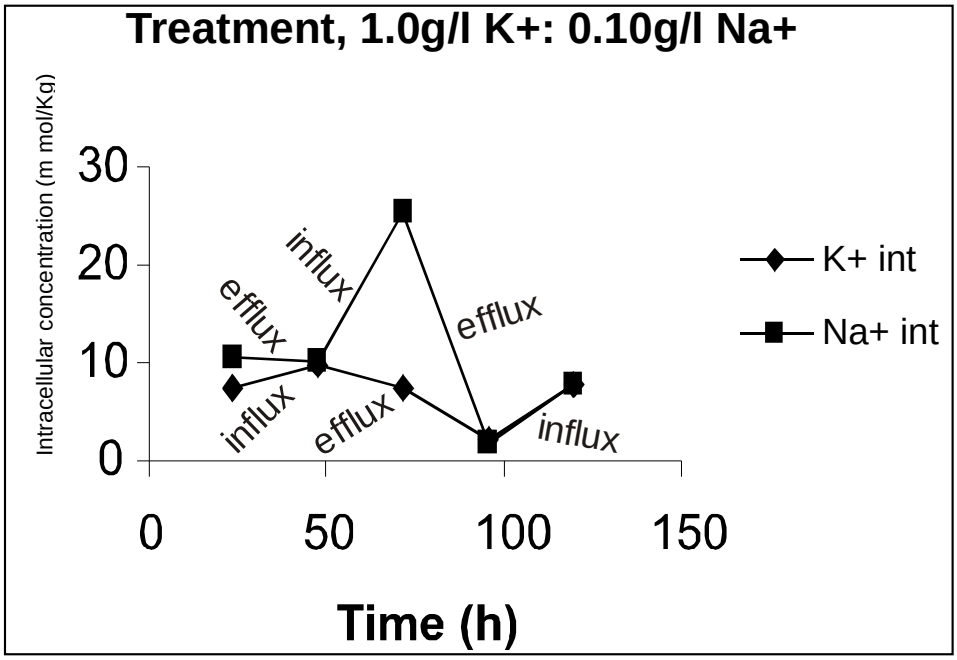

Figure 4. Graph showing the occurrence of ionic circulation between the bulk and intracellular media during the cultivation of *M. circinelloides* in synthetic broth.

contents increased with increase in Na^+ modulation and yeast form induction decreased oppositely, indicating that increase in intracellular Na^+ content suppressed yeast induction. Still, the proportion of yeast cells induced at the 1.10 g/l K^+ treatments was never as high as that which occurred at the 1.0 g/l K^+ treatment.

K^+ treatment, Na^+ influx and yeast induction

Generally, addition of Na^+ to culture broths stimulated the induction of yeast cells. At K^+ treatments where there was optimal intracellular Na^+ accumulations (0.9 g/l K^+: 0.05 g/l Na^+, 1.0 g/l K^+: 0.10 g/l Na^+, 1.10 g/l K^+: 0.15 g/l Na^+ and 1.10 g/l K^+: 0.20 g/l Na^+), the Na^+ influx rate also showed high values, but influx rate was lower with rise in Na^+ incorporation, and negative at 0.20 g/l Na^+ modulation, where intracellular accumulation of Na^+ was also the least (Supplementary Table 4).

This means that there was Na^+ extrusion at the log phase of growth in the latter treatments. Since observation showed that yeast cell induction was least with 0.90 g/l K^+: 0.05 g/l Na^+ modulation where Na^+ influx was highest, it probably means that Na^+ accumulation was not the sole determinant of yeast induction (from provident protoplast - to - formation of nascent yeast) at lag phase, and yeast proliferation (from matured yeast - to - terminal budding yeast) at log phase.

This was corroborated by the fact that optimal yeast induction occurred with treatment (1.0 g/l K^+: 0.10 g/l Na^+) intracellular accumulation of 17.95 mg/kg with an influx rate of 0.473 mg/h and, yet a sigmoid growth pattern exhibited. Still, the 1.0 g/l K^+: 0.20 g/l Na^+ treatment with Na^+ extrusion at log phase (- 0.0646 mg/h) and a 2.5 mg/kg intracellular accumulation of Na^+, supported sigmoid growth habit.

Perhaps, the lag phase composite entities, including germ cells, spheroid/-neoplasm, neoplastic units and protoplasts (which progressively increased in sizes), was also contributory to yeast induction.

Na^+/K^+ ratio, yeast induction and sigmoid growth pattern

At the K^+ (1.0 g/l) treatment that supported near perfect sigmoid growth habit, Na^+ modulation stimulated yeast proliferation. The Na^+/K^+ ratio was 2.9 at the yeast proliferative stage in the treatment combination; 1.0 g/l K^+: 0.1 g/l Na^+ (Figure 7).

Treatment combinations with Na^+/K^+ ratios approximating this sigmoid curve-associated ratio promoted high yeast inductive/proli-ferative capabilities no matter whether they exhibited a single, sigmoid or bi-phasic growth habit, although the relative proportions varied. Again, high Na^+/ K^+ ratio at log phase was not the sole determinant of yeast proliferation, for at 1.0 g/l K^+: 0.20 g/l Na^+ treatment combination, the Na^+/K^+ ratio at log

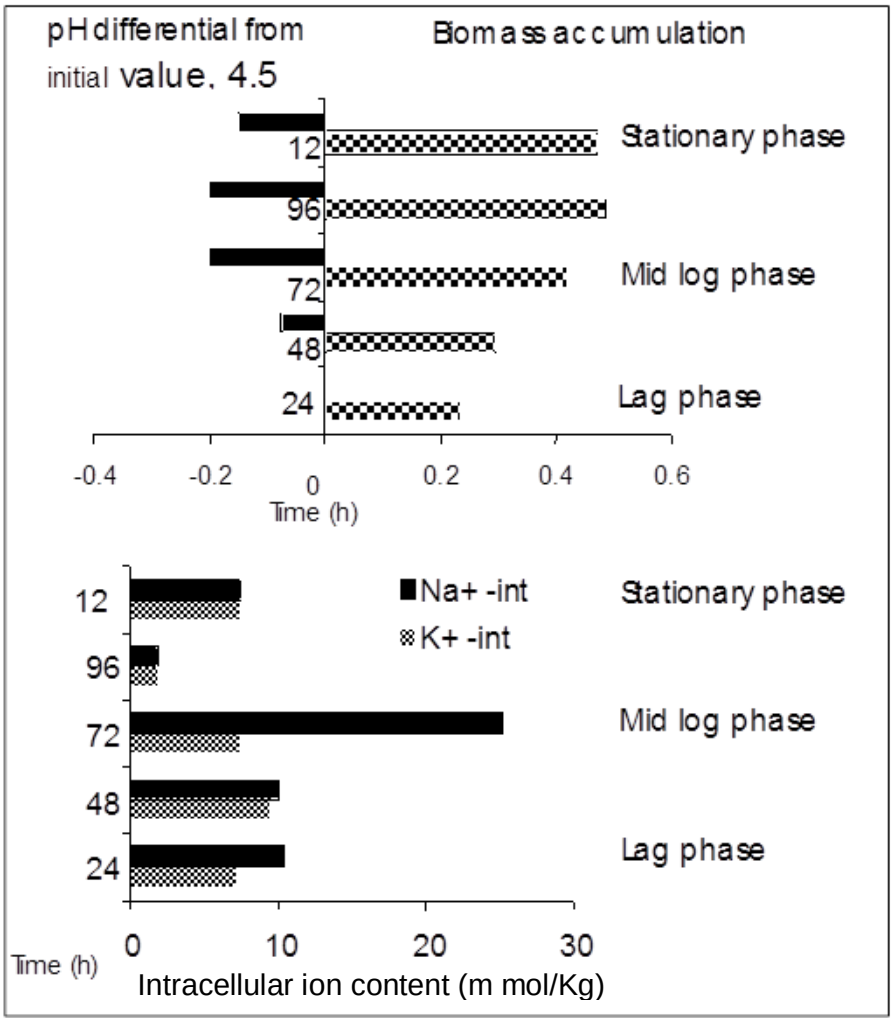

Figure 5. Histograms showing biomass accumulation and bulk medium pH variation in relation to intracellular ion content.

phase was comparatively low (0.56) yet growth habit was sigmoid and yeast cells preponderant.

However, in the 1.0 g/l K^+: 0.20 g/l Na^+ treatment Na^+/K^+ ratio at lag phase, 0.81, was higher than that at the primary sigmoid habit expressive treatment (0.78) (1.0 g/l K^+: 0.1 g/l Na^+).

Although there was efflux of K^+ at lag phase in the former, in contrast to its intracellular accumulation at the primary sigmoid habit expressive treatment, yeast cells still sequentially induced. As microscopic examination showed in the first set of experiments reported above, and in comparison with the other K^+ treatments, the 1.0 g/l K^+ treatment stimulated the induction of a higher proportion of yeast cells. This was further enhanced with the incorporation of Na^+ in such K^+ supplemented media (second set of experiments).

Following this, the proportion of yeast cells in broth peaked at 0.10 g/l Na^+ modulaion. In a similar manner, intracellular accumulation of Na^+ was higher at the 1.0 g/l K^+ treatment and it reached a peak at 0.10 g/l Na^+ broth modulation, in comparison with other Na^+ - levels. This indicated that the presence of Na^+ intracellular in particular, rather than K^+, stimulated the yeast form, thus confirming the results on biomass analysis shown in Table 3 and deductions made from Figure 1a. Therefore, if we use Na^+ influx rate as a parameter for measuring yeast induction (meq of Na^+ accumulated/time at specific growth phase, lag or log), we find that the value of rates was parallel with increase in intracellular Na^+ accumulate and consequently yeast induction. It was however not sequentially when it was viewed against Na^+ modulation in the minimal medium.

Since K^+ accumulation appeared to trigger lag phase - restricted neoplasm-protoplast generation and, Na^+ accumulation triggered vegetative capability, that is, from the provident protoplast through prevegetative- to vegetative

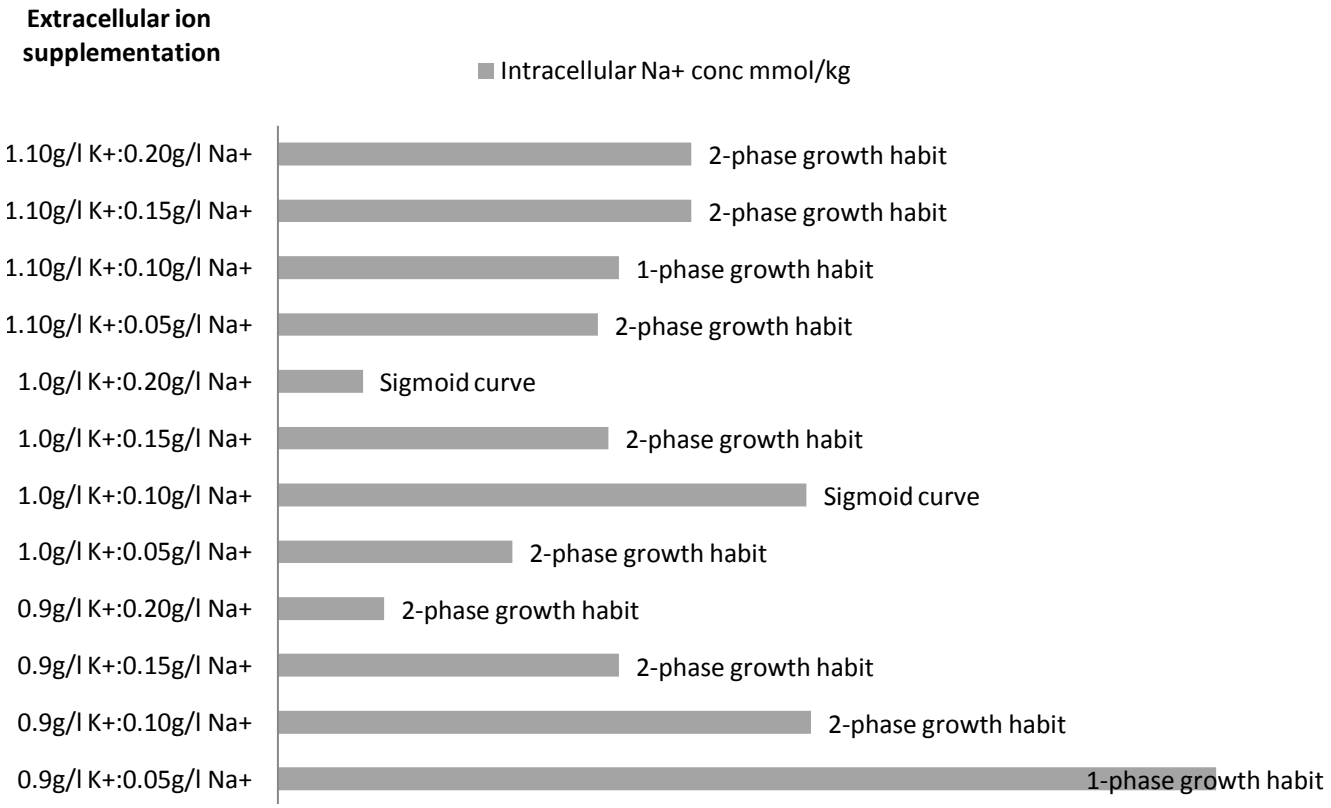

Figure 6. Histogram showing Na^+ accumulation after 72h of growth and growth patterns exhibited by *M. circinelloides* cultivated in synthetic broth.

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cell, the yeast form acquisition, which was both lag phase and log phase event, and in each phase both ions were antiported in a sigmoid-oriented growth pattern; it possibly meant that Na^+/K^+ ratio could be a more valid means of assessment of yeast induction, that is, at both the inductive stage and the proliferative stage in this study.

This also means that it was not the absolute levels of K^+ or Na^+ at this inductive stage, that is, lag phase, but the cellular Na^+/K^+ ratios that were the main determinants that promoted the induction of the yeast morphology. If we realized that the inductive stage encompassed several transient forms, including germ cells, spheroids/neoplasm, neoplasts, protoplasts, prevegetative cells (Figure 8) then the Na^+/K^+ ratio progressively impacted on these forms in the unidirectional milieu, that is, during the H^+ extrusion phase. Thus, as the H^+- release intensity increased against a concentration gradient in the bulk medium, being buffered at a specific acidic pH-level and hence proton ion gradient, there was anti-parallel decrease in the magnitude of Na^+/K^+ ratio: from high in the bulk medium to low intracellular. The Na^+/K^+ ratio at lag phase at which yeast induction was stimulated varied from 0.41 - 2.93 in the treatment combinations 0.90 g/l K^+: 0.05 g/l Na^+ to 1.10 g/l K^+: 0.20 g/l Na^+ (Figure 7).

Deviation from optimal Na^+/K^+ ratio and thallic expression

At lag phase, it appeared that a deviation from the sigmoid curve permissive Na^+/K^+ ratio, (0.78), inchoately enabled the occurrence of other morphological structures. Thus, with high Na^+/K^+ ratio, (2.93) at 1.10 g/l K^+: 0.10 g/l Na^+ treatment, polarization of germ cell could be triggered, prolongation of which gave rise to thallic growth, which initially could be coenocytic, determinate and subsequently thallo-arthric on the one hand, and septate which could give rise to conidiophore with apical vesicle bearing concurrent catenate conidia, on the other.

Variation in thallic expression occurred as germ tube converted to conidiogenous structure, which produced conidia in an acropetal manner, or holoblastic conidia as all the walls of a growth sphere participated in septum formation. The higher Na^+/K^+ ratio at lag phase possibly gave rise to the vigorous thallic growth at this treatment (1.10 /l K^+: 0.10 /l Na^+), in comparison with the others, and made the thallic subtypes more robust, as already

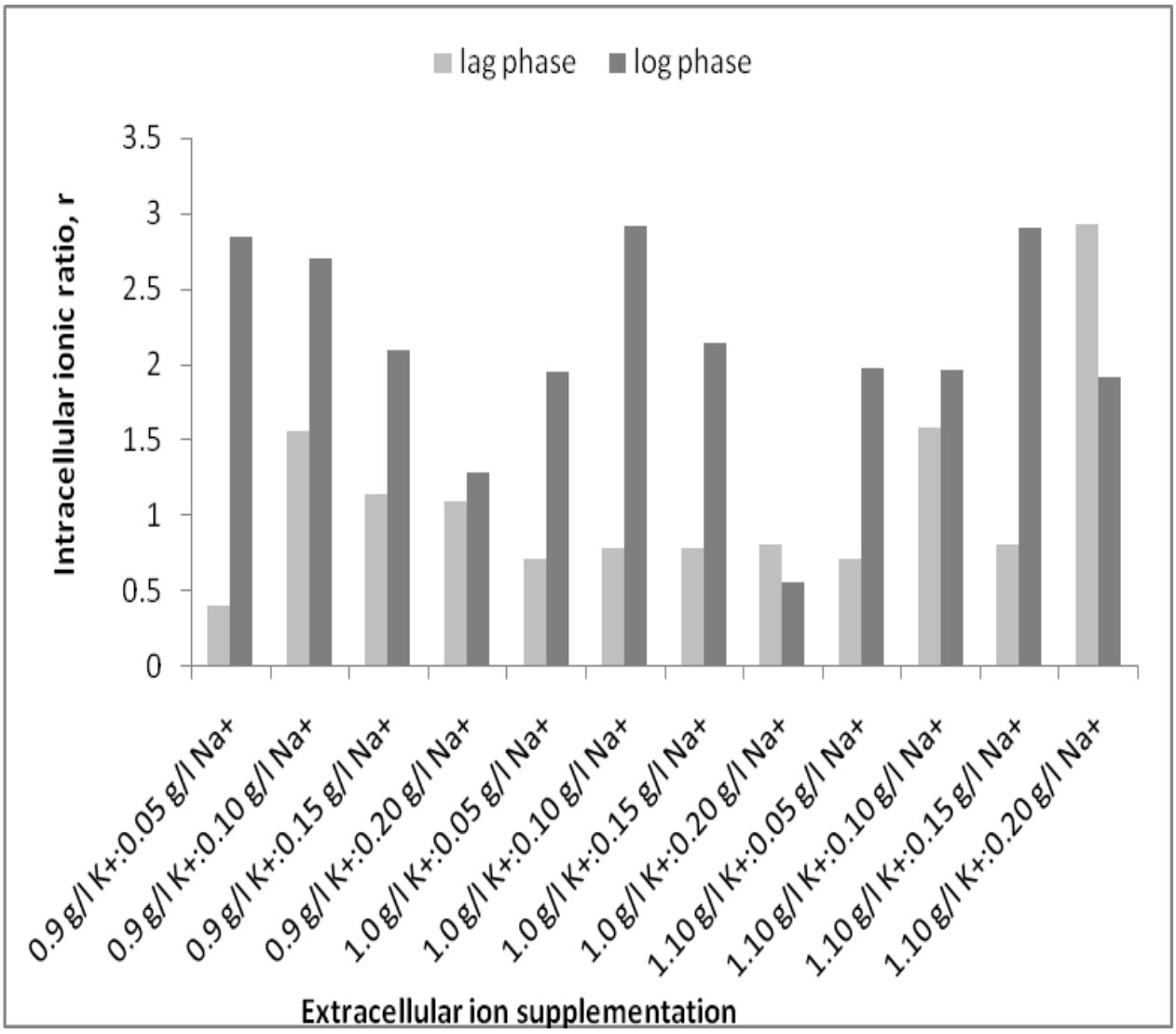


Figure 7. Treatment combinations and intracellular Na^+/K^+ ratio (r) in inductive (lag) and proliferative (log) phases during the cultivation of *M. circinelloides* in synthetic media.

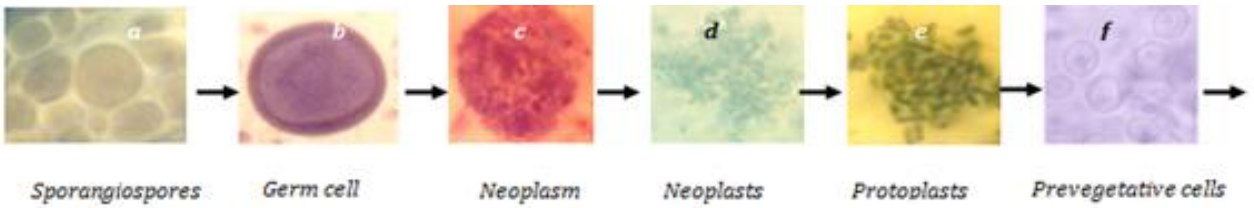
observed above.

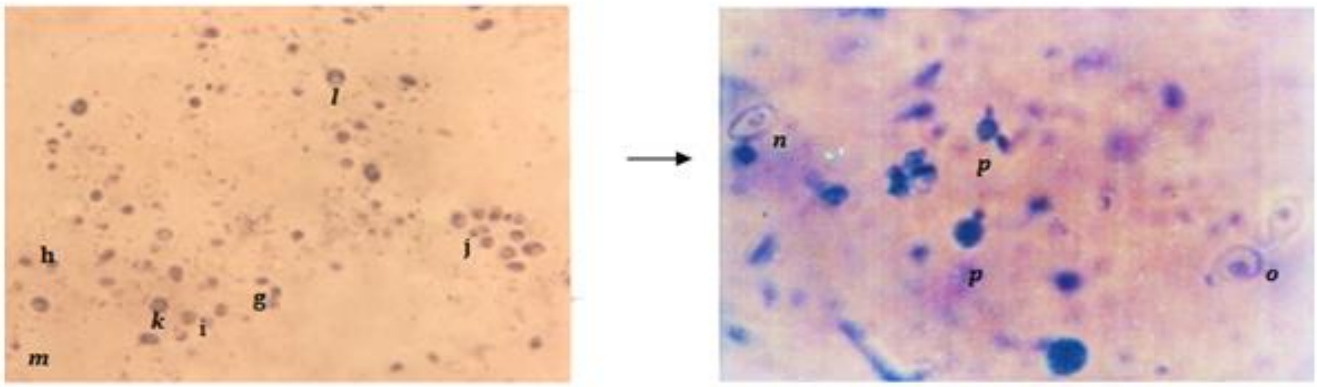
These persistent conidia remained in the growth milieu till termination of experiment. However, since it was presumably affected by the decreasing time-dependent Na^+/K^+ ratio, protoplasm of growth sphere or, conidia converted to neoplasm. Perhaps, as a result of internal pressure, witness conidia burst or cell wall rupture, thereby releasing neoplastic units or protoplasts. These thereafter assumed individual life form.

Ionic circulation and sigmoid growth phases

In the 1.0 g/l K^+: 0.10 g/l Na^+ treatment combination where there was optimal induction of terminal budding yeast cells, growth profile showed a sigmoid curve. This is shown in Figure 9. Demarcation of the growth phases also correlated with cryptic phases of ionic flux as shown in Figure 4, a feature anchored on transversal through the biomembrane, an indication of involvement of electrical signaling and free energy generation inherent in the concentration gradients of Na^+ and K^+ created across the membrane of the growing microorganism. At lag phase (Figure 9a), K^+ accumulated intracellularly as Na^+ decreased in a time dependent manner (24 - 48 h) (Figure 4), indicating a fall from high action potential to a low level across the membrane.

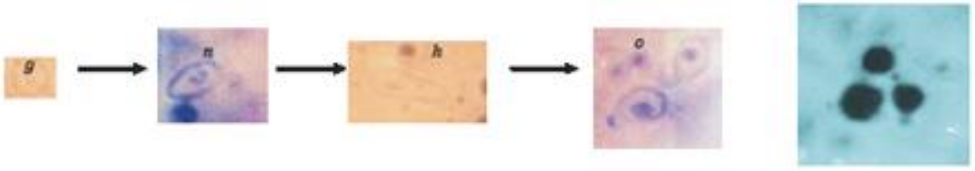
Starting with sporangiospore (Figure 8a), it coincided with the transition from germ cell through neoplasm, then neoplasts to protoplasts (Figure 8b to e), a clear





*Prevegetative cells & Nascent yeast: **g**=prevegetative cell, **h**=polarized prevegetative cell, **i**=pyriform nascent yeast, **j**=ovoidal nascent yeasts, **k** =globose nascent yeast, **l**=subglobose nascent yeast, **m**=obpyriform nascent yeast.*

*Prevegetative & vegetative cells: **n**=polarized prevegetative cell, **o**= nascent yeast, **p**= terminal budding obpyriform yeasts.*



Nascent yeast formation: observe as prevegetative cell membrane (**g**) becomes polarized (**n**) and elongates on its developmental axis (**h**) thereafter primarily becoming obpyriform (**o**) as inherent cellular components localized at the apex while the apparent nucleus is at mid cell.

Single & bipolar budding log phase obpyriform yeast cells.

Figure 8. Lag phase and log phase morphological expression of *M. circinelloides* cultivated in 1.0 g/l K^+: 0.10 g/l Na^+- treatment broth in this study; **a-f**, x2000., **g-m**, x 800 ., **n-q**, x1000.

demonstration of phenotypic modification at the lag phase.

At exponential growth phase (Figure 9b), the extrusion of K^+ observed and rapid intracellular accumulation of Na^+ (48 - 72 h) (Figure 4) as the emerging prevegetative cell (Figure 8f) subsequently assumed autocatalytic (from protoplast-to-prevegetative-to-vegetative) growth. This is further illustrated in Figure 8g to o which showed a wall-less membrane bound pre-vegetative cell become polarized at one locus, then it became directionally elongated on its axis until a wall-less vegetative cell initiate was formed. This indicated that through the period

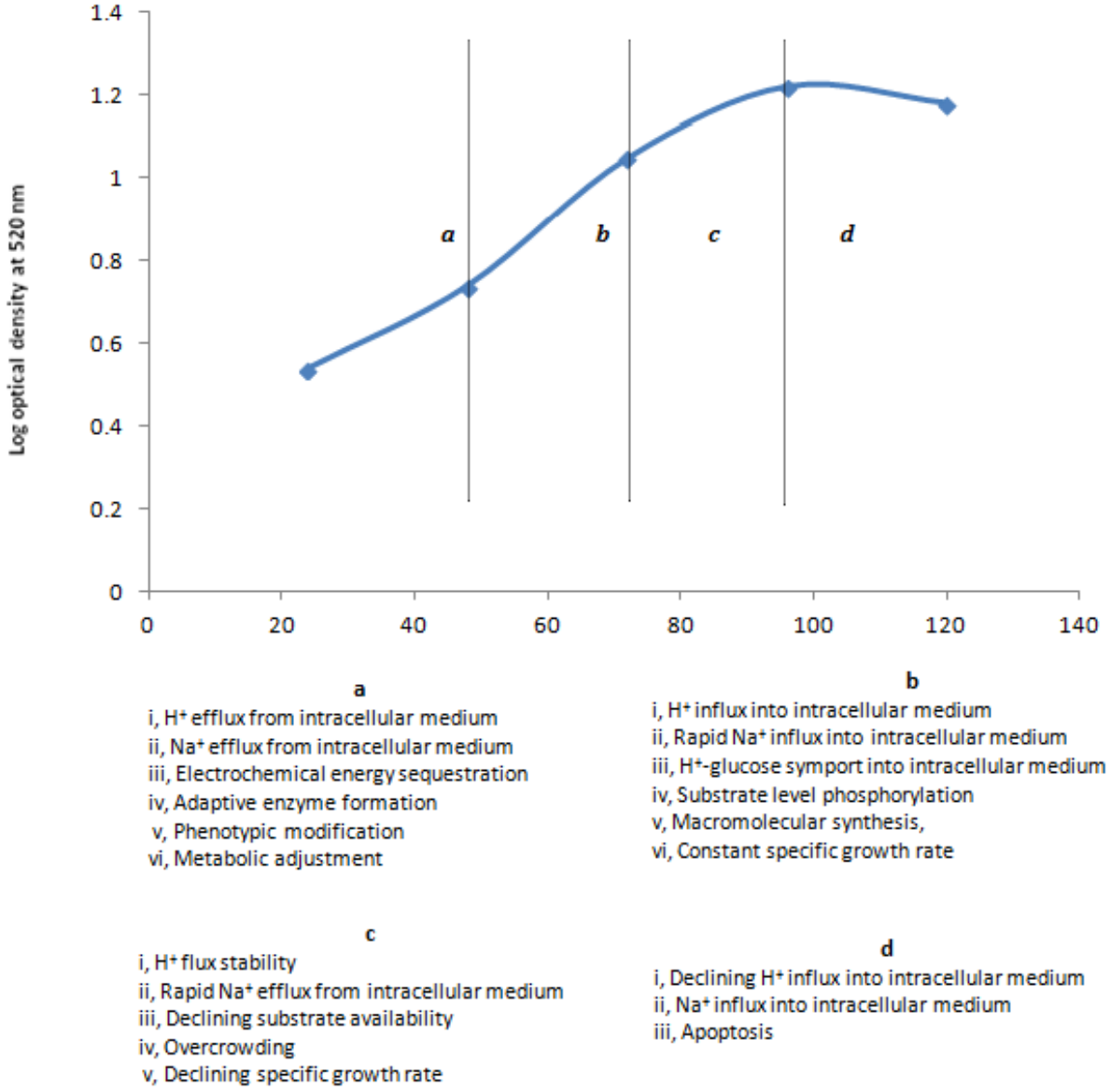


Figure 9. Sigmoid curve and possible electrophysiological processes that occurred in induced yeast cells of *Mucor circinelloides* when cultivated in glucose-substrate 1.0 g/l K^+: 0.10 g/l Na^+ broth at pH 4.5 and at 20°C.

of linear growth, biomebrane of induced yeast cells became more permeable to Na^+ as the vegetative cell matured and subsequently budded; hence there was rapid influx and intracellular accumulation of the ion and perhaps, its inherent use in driving bioenergetic and biosythetic activities, including cell wall construction, increasing macromolecular syn-thesis and mitosis thus leading to yeast cell proliferation (Figure 8p and q), marked by constant specific growth rate, and hence the exponential growth phase (Figure 9b).

In this study, the period of declining logarithmic growth (Figure 9c) coincided with very rapid extrusion of Na^+ and K^+ from the intracellular medium (Figure 4) (72 - 96 h). This also coincided with the period of pH stability (Figure 5a). It could also be said to be the period of nutrient exhaustion, overcrowding and toxic product accumulation. If this was assumed, then that there was pH stability probably meant that a steady state condition was created between the bulk and intracellular media for a biochemically redirected programme and, or divergent physiological activity which occurred intracellularly. Here, notice that rapid Na^+ extrusion from intracellular medium occurred, an indication of membrane repolarization arising after a possible log phase-associated membrane

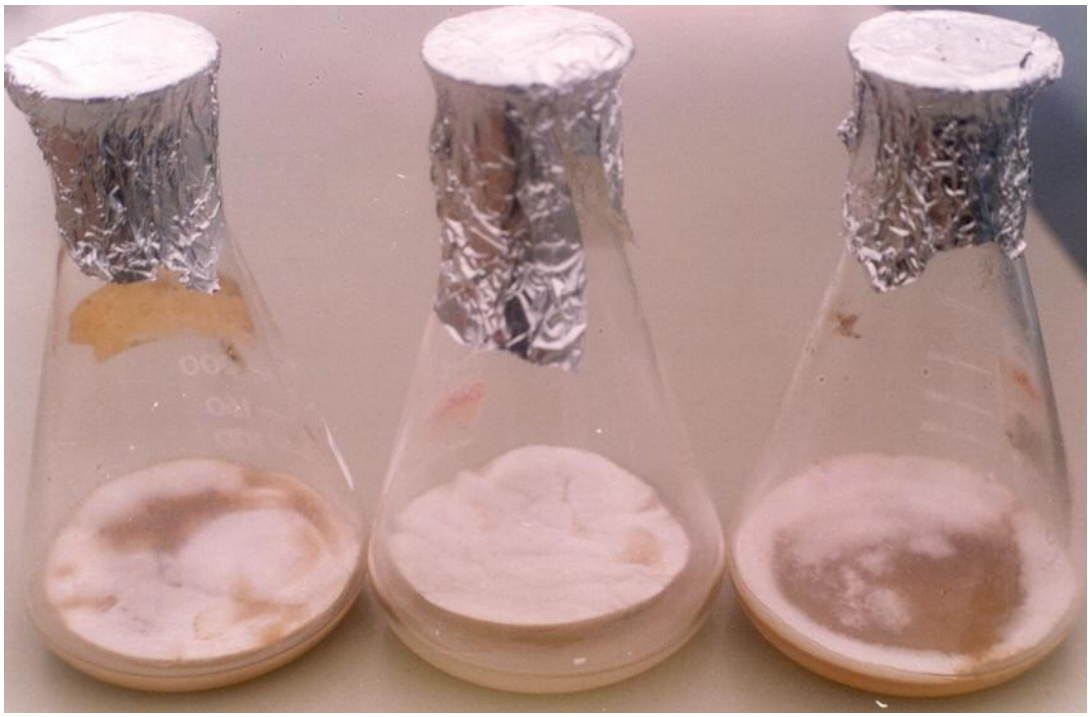


Figure 10. Flask-cultures of induced yeast cells of *M. circinelloides* after 2 weeks of growth. At termination of experiments conducted at pH 4.5, 20°C, the flasks were transferred to the laboratory side bench at 28±1°C, ambient. the reversion to aerial mat that was re-induced in each of the flasks was observed following transfer of cultures of induced thallic subtypes and terminal budding yeasts, which sedimented, to the laboratory side bench.

depolarisation in the induced yeast cell, which perhaps should normally incur a rise in membrane potential at this declining specific growth rate phase, but simultaneously there was a rapid or heightened K^+ efflux from intracellular medium in this anisotropic environment but in a segment where the pH steadied. That there was K^+ efflux at exponential growth phase, but this was further heightened at declining specific growth rate phase, perhaps indicated that membrane K^+ conductance played a more significant role in maintainng the upward trend of specific growth rate, but beyond optimum level, or threshold, that is, of conductance perhaps in the steady state, such support broke down and the specific growth rate set on a decline, a possibility that led to the assumed diverted biochemical and, or physiological event. Ionic transport into the intracellular medium was again unidirectionally reversed (Figure 4) (96 to 120 h) at the stationary phase (Figure 9d), where there was no further increase in the population level, but the assumed apoptotic programme set it on a steep fall.

Reversion to filamentous growth habit

At termination of experiments, culture flasks of induced yeast cells and thallic subtypes were shifted to the laboratory bench. Observation after 2 weeks of such transfer showed that filamentous growth formed compact matt on the surface of each culture broth. This is shown in Figure 10.

DISCUSSION

This study shows that *M. circinelloides* exhibited a multifaceted growth habit in synthetic broth, exhibiting both transient and stable morphologies. That neoplasm, derived from plasm of germ cells reorganized into multiple individual units, including neoplasts and protoplasts, which were not observed in the control tests except when K^+ was incorporated, confirmed finding that K^+ was of absolute necessity for protoplast formation (Omoifo, 2012). As it has been shown in several studies, a sequential development occurred with the protoplast being the cross-over point of a process that led to the emergence of the yeast form (Omoifo, 2003, 2009). This was confirmed in this study. This study's finding differ from that of earlier workers (Lubberhusen et al., 2003; McIntyre et al., 2002) who obtained multipolar budding yeastlike cells from dimorphic *M. circinelloides* under 30%

CO_2 pressure but thallo-arthric conidia on oxygen stimulation.

Yeast induction has been attributed to three main parametric effects, viz: critical concentration of ions, chemical potential and Na^+ influx rate; these combined in a cooperative manner to effect a streamlining of the phenotypic expression (Omoifo and Awalemhen, 2012). In the present study, the concentration of ions has been found to be most significant in the early process of induction and subsequent proliferation of the induced yeast form.

This has been expressed as the Na^+/K^+ ratio. While a diminishing magnitude of this parameter, from the bulk medium to the intracellular medium led to reorganization of the stable spore-morphology through cryptic transient forms, it was thereafter reversed after the cross-over form, that is, the protoplast, and with an increasing effect until a stable phenotype was achieved. This was the yeast cell. However, the Na^+ influx rate assumed greater significance in expounding the yeast form differentiation, from protoplast through prevegetative cell-to-nascent yeast, then through maturation until it became terminal budding.

Thus, in a sigmoid growth expressive habit, as occurred in the 1.0 g/l K^+: 0.10 g/l Na^+ treatment, a lag - log sequential parametric relationship, or cooperativity was essential for the yeast form induction. Lysed germ cell envelop, thereby 'freeing' the individual neoplasts, which dispersed within the medium, was the main method of generation of these units.

Beside this, a secondary type of ontogeny was when hyphal compartment ruptured or conidium burst, whence encompassing wall remained as carcass or ghost cell, an effect seen mainly at commencement of the 2nd phase of the pH profile involving oppositely unidirectional H^+ influx (Omoifo, 2012; this study), which (conidial burst or wall rupture) could have resulted from osmotic effects and, or an increase in turgor pressure within cellular compartments. The reversal in direction in H^+ transport also involving membrane bound transport mechanism and, hence Mitchellian proton pump (Haris, 1977; Voet and Voet, 1995) directed into the protoplast interior, would affect other radicals concentration, including phosphate group (Lehninger, 1975). Na^+ and K^+ as demonstrated in this study, thus confirming the earlier findings (Omoifo and Awalemhen, 2012) on intermedial ionic circulation, and consequently alter the inherent physiology (Calcot, 1981). But in a carbon-substrate growth milieu, this led to H^+-substrate symport (Alderman and Hofer, 1981; Mitchell, 1967; West and Mitchell, 1972, 1973, Slayman and Slayman, 1977) into the protoplast interior, thus providing glucose intracellular as used in our study, for bioenergetics and biosynthetic purposes.

At Na^+ efflux, there was simultaneous influx of K^+, a condition reversed at 2nd phase of the pH profile, when protons were translocated into the cellular interior, and as found in *Escherichia coli,* driven by the membrane potential generated as a result of this reverse movement (Setty et al., 1983) which corresponded with initialization of polarization of prevegetative cell, formation of nascent yeast and subsequent yeast proliferation in the study. Now, since K^+ is exchanged for H^+ mediated by membrane-bound proton pumps (Conway and O'Malley, 1946; Rothstein and Enns, 1946; Pena, 1975; Ryan and Ryan, 1972; Albert et al., 1994), then presumption of an anaerobic growth environment in a medium mediated by unidirectional H^+ influx into protoplast cytosol, and based on Mitchell's hypothesis on H^+ translocation in the absence of oxygen (Mitchell, 1979), would call for further inductive enzymes for a directional physiology; for instance, a reduction in Fe^{3+} is a condition that favored glycolytic breakdown of phosphate esterified glucose at carbon 6 (Michelson, 1978). Phosphoglucomutase, an enzyme that converts phosphate esterified glucose to glucose-1-phosphate, has been found in higher proportions in the yeast cells of dimorphic *Paraccoccidioides brasiliensis,* in comparison with the mycelial form (Kanetuna and Carbonell, 1966). Glucose-1-phosphate is a key intermediate in the pathway to the formation of beta-1, 3 glucan, the key structural material of the yeast cell wall. Assuming that these activities occurred during the growth of *M. circinelloides*, from this, it can be seen that the possible induction of the enzyme phosphoglucomutase could lead to conversion of co-H^+-symported glucose to the yeast cell wall structural material, a platform for formation of nascent yeast (Figure 8i-m) copiously observed in this study.

The cell wall generative capacity of protoplast is not in doubt, as Carbonell et al. (1973) showed that the protoplasts of dimorphic *H. capsulatum* prepared by enzymatic lysis of cell wall from whole yeast cell, could re-generate the cell wall. It could be inferred from the above discussion that catabolic breakdown of the presumed H^+-symported glucose within the protoplast was glycolytic. This has been demonstrated in our laboratory (Omoifo et al., 2013) by the use of the well-known glycolytic inhibitor, NaF, which exhibited three main effects on the conversion process of sporangiospores of *Rhizopus stolonifer* to terminal budding yeast cells in minimal medium, viz: (a) complete inhibition of yeast induction, (b) delayed induction of yeast cells and (c) apoptosis of induced yeast cells. In the complete absence of yeast cells, protoplasts were copiously produced. When yeast cells were induced prior to NaF challenge, the inhibitor caused the death of cells. It was strongly suggested that NaF challenge had profound effect on substrate level phosphorylation, which is primarily executed at the enolase enzyme activity step, where NaF irreversibly combines with the enzyme thereby preventing it from performing the significant role of enolization of phosphorglycerate to pyruvate thereby terminating substrate level phosphorylation, being that fluoride ion forms a complex

with Mg^{2+} that competitively locates the Mg^{2+}-requiring enolase active site (Cinnasoni, 1972), an effect enhanced with high levels of phosphate (Bassetti et al., 2004) also copiously used in our medium, (note that Mg^{2+} was part of elemental composition of our multiionic system in the present study), and thus prevents the dehydration of 2-phosphoglycerate and, hence the formation of phospho-enolpyruvate (Voet and Voet, 1975; Christophe et al. 2001), as this is a critical step in the generation of ATP through substrate level phosphorylation. This implied that protoplasts through prevegetative cell per-formed energy generation through the physiological mechanism of substrate level phosphorylation, which could be terminated with the glycolytic inhibitor. But when the inhibitor challenge was prior to generation of a functional envelop, the transient forms remained as protoplasts.

It is demonstrated here that the exhibition of sigmoid growth curve by induced yeast cell of *M. circinelloides* is similar to the life pattern of *Saccharomyces cerevisiae*. It is demonstrated here also that a classical sporangiospore-producing filamentous microorganism, *M. circinelloides*, converted through multiple transient forms debuting as prevegetative cell and subsequently proliferating neither as sporangiate thalli nor conidiate thalli, but terminally buds unicellular cells, which describe a sigmoid growth pattern. Such should have properties that could be used for comparative purposes. This study confirmes earlier report (Omoifo and Awalemhen, 2012) that *M. circinelloides* exhibited sigmoid growth when 1.0 g/l K^+ treatment was modulated with either 0.10 or 0.20 g/l Na^+ but with higher specific growth rate in the former. It was also shown that broth where the exponential growth phase was more steeply oriented permitted a near perfect sigmoid curve at 1.0 g/l K^+: 0.10 g/l Na^+ treatment. An earlier study (Omoifo, 2012) showed that the H^+-released intensity from intracellular medium of the growing microorganism was maximal in the 1st phase of the pH-profile, that is the extracellular medium reached the maximum degree of acidification. This is akin to increase in external medium pH changes when *Escherichia coli* is suspended in acidic medium whereby the internal pH approaches neutrality (Padan et al., 1976; Booth et al., 1978, 1979; Zilberstein et al., 1979), a system that is energy dependent (Padan et al., 1976; Mitchell, 1979; Setty et al., 1983). As the present study shows, this was when Na^+ ions were correspondingly extruded from the intracellular medium. In the 2nd phase of the pH-profile when there was diminishing proton-release intensity (Omoifo, 2012; this study), the Na^+ influx rate was optimal, which was 0.47 mg/h for that treatment in comparison with the 1.0 g/l K^+: 0.20 g/l Na^+ treatment, the less permissible sigmoid curve-oriented growth-medium where the Na^+ influx rate was 0.12 mg/h (Omoifo and Awalemhen, 2012). The lower sodium influx rate also therefore appeared to correspond with lower proton ion electrochemical potential. As this study shows, the difference in sodium accumulation and inherent proton potential was also reflected in the yeast induction capability of the media as the former (1.0 g/l K^+: 0.10 g/l Na^+ treatment) induced comparatively higher population level of terminal budding yeast cells.

That the biphasic pH profile was similar whether growth was sigmoid or 2- optima habit or the variant was of either, suggested that physicochemical activities permissible with transmembrane- proton ion - gradient were also similar during the patterned growths. However, because of imbalance in ionic content at lag phase, that is, deviation from the treatment 1.0 g/l K^+: 0.10 g/l Na^+ ratio (0.78), biochemical activities possibly promoted localized polarization of growth spheres; tubular growth therefore ensued. This was probably the origin of the thallic subtypes observed in this study. Although, inherent biophysical phenomena took place and these included neoplasm formation, cell envelop rupture and release of neoplastic units, protoplasts formation, wall-less prevegetative cell formation and its polarization until a wall-less vegetative cell initiate was formed, which were all lag phase events.

Since lyses of cell wall of thallic subtypes were not complete, carcasses of same were conspicuous and they became additional to biomass quantitation at the lag phase. Hence they contributed to the first optimum of the 2- phase growth habit. As it was obvious, post cell envelop rupture modifications of protoplasts were subjected to the same ionic effects as occurred in the sigmoid- related log phase that induced terminal budding yeast cells. This was possibly why terminal budding yeast cells were also induced, along with, and after the first phase thallic growth cessation, in the two- phase growth pattern and became more preponderant during the second phase.

Conclusion

In the morphological species concept, it does not appear that neoplasm, neoplast, protoplast, prevegetative cell and nascent yeast have been characterized for filamentous microorganisms. Specifically, transformation of spores of *M. circinelloides* has been significantly influenced by the establishment of transmembrane proton ion gradient and this led to demarcations resulting in transient forms observed in this study in which ionic circulation correlated with morphology. This thus suggested that the morphological, biochemical and physiological sequences herein described were tightly coupled to each other and thus illustrated a process that appeared dependent on self assembly with inherent membrane restructuring at key junctions but driven by a transmembrane proton ion gradient. It was argued here that, active reactions led to energy generation through the electrochemical proton gradient in phase 1 favouring

phenotypic modifications and this was sequentially followed by substrate level phosphorylation in the ultimately adopted morphological form, the yeast cell.

It further showed the significance of Na^+/K^+ ratio in determining the early growth phases of *M. circinlloides* in a sigmoid oriented growth pattern and deduced that a deviation from the sequential parametric relationship led to expression of morphologies other than the yeast form, which physiologically catabolized glucose-carbon through substrate level phosphorylation; but at the stationary phase, perhaps a diminished effect of the Na^+/K^+ ratio led to equal generation and subsequent apoptosis of the induced terminal budding yeast cells. Thus, it could be surmised that the process of yeast induction from aerially borne spores of *M. circinelloides* occurred in a highly functional proton gradient driven cooperative system in which modulation by phase-determining sodium-potassium ionic ratio became significant. But exposure of such induced yeast to atmospheric oxygen led to a reversion to the filamentous growth habit.

Although this study is only exploratory, it offered opportunity to view the issue of fungal dimorphic switching with a consideration given to electrophysiological relationships. The specific effects of Na^+ and K^+ however could not be taken as absolute as discrepancies exist. For instance, in the study of Omoifo and Awalemhen (2012), it was shown that at the declining specific growth rate phase K^+ mode was promoted, which is incongruous with the present deposition. However, if this study suggested a transmembrane proton ion gradient as another paradigm for dimorphic switching of *M. circinelloides*, then the dynamics of K^+ conductance on membrane activation, lysis of germ cell envelop and the process of polarization of prevegetative cell, as well as it's possible effect on Na^+ conductance and inherent organelle mobilization on one hand, and yeast cell proliferation on the other, call for closer attention, especially as Setty and co-workers (Setty et al., 1983) showed that the relative concentration of K^+ and membrane permeability are major factors that control the kinetics and degree of H^+ movement.

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Supplementary Table 1. Analysis of variance of growth data of *M. circinelloides* cultivated in buffered synthetic broths with different K^+ level incorporation.

Source of variation	d. f.	Sum of squares	F-value
K^+- level	5	1.14982	0.042*
Time	4	0.48773	0.256
K^+ -level x time	20	2.52804	0.172
Residual	30	2.60395	
Total	59	6.76954	

*F-value significant at $p<0.05$.

Supplementary Table 2. Analysis of variance of growth data of *M. circinelloides* cultivated in buffered synthetic broths with different K^+ incorporation.

Source of variation	Degree of freedom	Sum of squares	F-calculated
Rep. stratum	1	0.1403	
K^+-level	2	0.0097	0.59
Na^+-level	3	0.0915	0.025*
Time	4	0.5248	<0.001*
K^+-level x time	6	0.0287	0.78
K^+-level x Na^+- level	8	0.0321	0.89
Na^+-level x time	12	0.1244	0.35
K^+-level x Na^+-level x time	24	0.2026	0.57
Residual	59	0.5401	
Total	119	1.6946	

*F-value significant at $p<0.05$; lvl = level.

Supplementary Table 3. Regression analysis of response variate, optical density, on K^+ and Na^+ supplementation and accumulation.

Source of variation	Degree of freedom	Sum of squares	F-calculated
Regression	4	0.088	0.86*
Residual	115	1.607	0.591*
Total	119	1.695	

F-value not significant at $p<0.05$.

Supplementary Table 4. Treatment combinations and Na^+ and K^+ influx rates into the cell during the growth of *M. circinelloides* in synthetic broth.

Treatment	K^+ influx	Na^+ influx
0.9 g/l K^+: 0.05 g/l Na^+	-0.325	1.2625
0.9 g/l K^+: 0.10 g/l Na^+	0.0413	0.0667
0.9 g/l K^+: 0.15 g/l Na^+	-0.375	0.0729
0.9 g/l K^+: 0.20 g/l Na^+	-0.0083	-0.2146
1.0 g/l K^+: 0.05 g/l Na^+	0.0146	0.0625
1.0 g/l K^+: 0.10 g/l Na^+	0.0896	0.4729
1.0 g/l K^+: 0.15 g/l Na^+	-0.0458	0.1625
1.0 g/l K^+: 0.20 g/l Na^+	-0.0708	-0.0646
1.10 g/l K^+: 0.05 g/l Na^+	-0.2333	0.333
1.10 g/l K^+: 0.10 g/l Na^+	-0.1167	0.1583
1.10 g/l K^+: 0.15 g/l Na^+	0.077	0.425
1.10 g/l K^+: 0.20 g/l Na^+	-0.325	0.4125

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Full Length Research Paper

The biodiversity of oleaginous microalgae in Northern Qinghai-Tibet Plateau

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Microalgae represent an exceptionally diverse but highly specialized group of micro-organisms that adapt to various ecological habitats. Due to their unique environment (extremely low temperature and anoxia), the Tibetan Plateau is among the regions with numerous rare ecotypes such as arid desert, salt marsh, alpine permafrost, hot spring and lawn. In this study, 52 microalgae from different environments in Qinghai-Tibet Plateau were isolated and divided into 11 different groups based on the results of restriction fragment length polymorphism (RFLP). Then, 2-3 strains of each group were selected for 18s rDNA molecular identification. The oil contents among the studied strains varied from 3.26 to 25.47%. The results indicate that the number and high lipid content of the strains isolated from the water were much more than those from the soil. This provides a direction for the isolation of high oleaginous microalgae.

Key words: Northern Qinghai-Tibet Plateau, microalgae, lipids, restriction fragment length polymorphism (RFLP), 18s rDNA.

INTRODUCTION

Conventional biodiesel mainly comes from soybean and vegetable oils (Huang et al., 2010; Bunyakiat et al., 2006), palm oil (Al-Widyan et al., 2002), sunflower oil (Antolinet al., 2002), rapeseed oil (Peterson et al., 1996) as well as restaurant waste oil (Bouaid et al., 2007). Producing lipids by microbial fermentation is a new approach of lipids. Oleaginous microorganisms are now used more and more widely for producing functional oil and biodiesel. Among them, microalgae have many advantages such as high photosynthetic efficiency, fast growth rate, no restriction of season and climate, easy realization of large-scale production. Thus, microalgae are considered as raw materials for biodiesel with a lot of potential (Chisti, 2007). Exploiting oil resource with microalgae has attracted more and more attention. Biodiesel production with microalgae has become one of the important research areas.

Microalgae are photosynthetic micro-organisms that convert sunlight, water and carbon dioxide to algal biomass. Many microalgae are exceedingly rich in lipid (Chisti, 2007; Banerjee et al., 2002) which can be converted to biodiesel with the current technology.

At present, the research on oil microorganism focuses on exploring oil microorganisms by screening the existent microorganisms, or optimizing the microbial fermentation conditions to improve the lipid content, yet there are few research for isolation and diversity of the oleaginous microorganisms in different environments, which has limited the oil microorganisms' species. Northern Qinghai-

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Tibet Plateau is extremely cold and anoxic, which makes it one of the regions with special ecotypes.

As a result, abundant extreme microorganisms accumulate there. This special ecological environment includes high direct solar radiation, low atmospheric temperature, large diurnal temperature range, lot of salt lakes (Li, 2008), arid desert, salt marsh, alpine permafrost, hotspring, and lawn. In order to provide basic and cheap raw materials for the industrial production of microalgae lipid, a preliminary investigation has been done on the biodiversity of the oleaginous microalgae in Northern Qinghai-Tibet Plateau.

This experiment is mainly to study the oleaginous microalgae biodiversity in this region through isolation, identification, classification of the microalgae and the determination of the lipid content.

MATERIALS AND METHODS

Collection of samples

The samples including soil and water were collected from hot springs, wetlands, sands, grasslands, crop environment and saline, high radiation environment. Soil samples were collected at 5 - 20 cm under the surface, and immediately stored at 4°C.

Isolation, preservation and cultivation of microalgae

SE medium: $NaNO_3$ 0.25 g/L, $K_2HPO_4 \cdot 3H_2O$ 0.075 g/L, $MgSO_4 \cdot 7H_2O$ 0.075 g/L, $CaCl_2 \cdot 2H_2O$ 0.025 g/L, $KH_2PO_4$0.1175 g/L, NaCl 0.025 g/L, soil extract 40-50ml/L, $FeCl_3 \cdot 6H_2O$ 0.005 g/L, Fe-EDTA 1 ml/L, As Solution 1 ml/L and dissolved in synthetic seawater (Wang et al., 2010).

Isolation and preservation of microalgae

Petri dishes containing growth medium solidified with 1-1.5% agar medium were prepared. The agar was ½ to 2/3 the depth of the dish. 1-2 drops of mixed phytoplankton sample were placed near the periphery of the agar. A wire loop was flamed sterilize.

The sterile loop was used to make parallel streaks of the suspension on the agar by aseptic technique. 16 streaks (4 sets of 4) were made and the whole surface of the agar plate was used. The plate was covered and sealed with parafilm. And then was inverted and incubated under low light at constant temperature. Colonies free of other organisms were selected for further isolation. The sample was removed using a sterilized wire loop and placed in a drop of sterile culture medium on a glass slide. We checked microscopically to ensure that the desired species was isolated and is unialgal. The streaking procedure was repeated with the algal cells from a single colony and colonies were allowed to develop. This second streaking reduces the possibility of bacterial contamination and of colonies containing more than one algal species. Selected colonies were transferred to liquid or agar medium.

Cultivation of microalgae

Each microalgae strain was grown in 1 L Erlenmeyer flask containing 500 ml SE medium (pH 6.8) in a temperature controlled incubator at 25±2°C and 14 h light/10 h dark photoperiod.

Determination of lipid content

The isolated algae were inoculated into the SE medium, and then their lipid contents were determined under the same conditions.

Estimation of dry weight

Dry cell weight was determined gravimetrically according to Hu et al. (2008). A known volume of algal culture was centrifuged for 10 min at 5,000 rpm and the harvested biomass was dried in vacuum at 60°C; a constant weight was obtained.

Extraction of lipid from algal biomass

Extraction of lipid was done following the protocol of Bligh and Dyer (1959). Calculation of lipid content is as follows:

Lipid content = (Lipid weight/Dry cell weight) ×100% (Cao et al., 2004).

Restriction fragment length polymorphism (RFLP) analysis of the microalgae Extraction of DNA and PCR amplification of 18s rDNA

A microalga-specific forward primer18sF (5′GTCAGAGGTGAAATTCTTGGATTTA-3′) was used with reverse primer 18sR (5′-AGGGCAGGGACGTAATCAACG-3′ (Sara et al., 2009)) in order to obtain a 740 bp PCR product. The DNA was extracted using the cetyltrimethylammonium bromide method (Bellstedt, 2010); about 50 ng of DNA was used as template for amplification in PCR. Each PCR reaction consisted of a total volume of 50 µl containing 20 pmol of each primer, 200 µmol/L concentrations of each deoxynucelotide triphosphate, 1.5 mmol/L magnesium chloride, 0.15 mmol/L 10× PCR buffer, and 2.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). The PCR conditions were as follows: initial denaturation at 94°C for 5min, 30 cycles of amplification consisting of a 30 s denaturation step at 94°C, a 30 s annealing step at 56°C, a 1-min extension step at 72°C, and a final 10 min extension at 72°C.

RFLP analysis

PCR products (approximately 100 ng) were digested at 37°C for 2 h. Each digest contained 7 µl cleaned PCR product, 0.5 µl restriction enzyme *Hha* I, and 0.5 µl *Hae* III, 1µl restriction buffer and filled up to a final volume of 10 µl with ultrapure water. The reaction mixtures were electrophoresed at 3.5% agarose gel in a 0.5×TBE buffer system. Marker of 100 bp ladder (TaKaRa, Biotech. Co., Ltd., Dalian) was used as a size marker. The reaction mixtures were recorded with the digital imager (Bio-rad, USA) after staining with ethidium bromide (0.5 µg mL^{-1}). After analysis of each restriction map, a matrix analysis was done with the Ntrys software.

Cloning of 18s rDNA and sequencing

18s rDNA gene from each microalgae sample was amplified as described above and used for direct cloning with the TOPO-TA cloning system (Invitrogen, Shanghai) following the manufacturer's instructions. The ligated plasmids were transformed into *Escherichia coli* JM109 with high transformation efficiency. The transformed cells

Table 1. Isolation and lipid contend determination of microalga.

Strain no.	isolation medium	Environment	Content of lipids (% of the dry weight)	Strain No.	isolation medium	Environment condition	Content of lipids (%)
2	water	Wetland	17.67	34	water	Shaliu River	23.32
3	water	Wetland	17.81	35	soil	Wetland	15.74
5	water	Wetland	24.02	36	soil	Wetland	17.94
6	water	Wetland	9.84	37	soil	Grassland	16.08
7	water	Qinghai Lake	18.31	38	soil	Grassland	7.33
8	water	Qinghai Lake	16.84	39	soil	Firm ground	12.87
9	water	Wetland	14.98	40	soil	Rhizosphere soil	18.01
10	water	Qinghai Lake	16.35	41	soil	Sand	20.43
11	water	Qinghai Lake	8.62	42	soil	Wetland	17.96
12	water	QinghaiLake	20.83	44	soil	Wetland	25.45
13	water	Wetland	17.84	45	soil	Grassland	19.98
15	water	Qinghai Lake	25.17	46	soil	Rhizosphere soil	15.45
17	water	Qinghai Lake	25.47	47	soil	Salinate fields	17.42
18	water	Fairy Cove	16.57	48	soil	Grassland	21.88
19	water	Fairy Cove	14.29	49	soil	Wetland	8.53
20	water	Sewage treatment plant	12.41	50	soil	Wetland	13.24
21	water	Shaliu River	19.72	51	soil	Wetland	21.57
22	water	Shaliu River	22.89	52	soil	Firm ground	10.74
23	water	Wetland	14.99	53	soil	Hot spring soil	6.29
24	water	Wetland	3.83	56	soil	Frozen soil	14.89
26	water	Sewage treatment plant	11.37	57	soil	Firm ground	10.06
27	water	Fairy Cove	3.26	58	soil	Hot spring silt	22.02
28	water	Shaliu River	14.73	60	soil	Rape fields	6.47
29	water	Shaliu River	18.2	61	soil	Grassland	15.27
31	water	Sewage treatment plant	8.88	62	soil	Firm ground	12.02
32	water	Fairy Cove	13.14	63	soil	Lake side	20.96

were plated on Luria-Bertani agar plates containing 50 mg/µl Ampicillin. The positive recombinant clones were picked for direct PCR amplification with 18sF and 18sR primers. The clones were sequenced in company (Invitrogen, Shanghai) and the sequences were analyzed in NCBI database.

RESULTS

Isolation and lipid content determination of selected microalgae strains

Fifty two strains of oleaginous microalgae from a variety of habitats in Qinghai Province had been isolated; among them 27 strains were isolated from water samples and 25 strains from soil samples. The lipid contents of 52 strains were determined in the fermentation medium (Table 1).

There was a significant difference in the lipid content of all 52 strains. The minimum lipid content of 3.26% was strain No. 27 isolated from the fairy cove; the maximum was 25.47% corresponding to strain No.17 isolated from Qinghai Lake.

Among all analyzed microalgae samples, there were 12 strains (accounting for 23.1% of the 52 strains) with lipid content higher than 20%; and there are 19 strains (accounting for 36.5% of the 52 strains) with lipid content between 15 and 20%.

Diversity analysis of microalgae

Validation by restriction fragment length polymorphism (RFLP) method

According to the results of RFLP (Figure 1), all of the microalgae are divided into two groups. The first group contains the microalgae strain No. 17, 22, 26 and 34; the second group contains the microalgae strain No. 2, 8 and 28. The PCR products of the above mentioned seven microalgae strains were digested by the restriction enzyme Hha I and Hae III.

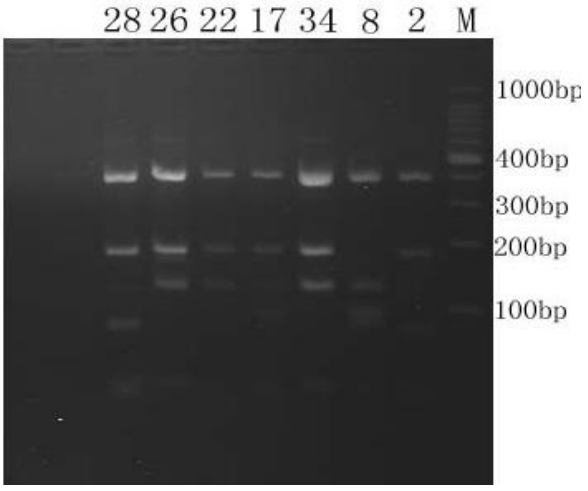

Figure 1. 18s rDNA fragment bidigestion results of seven microalgae LaneM:100bp DNA Ladder Marker.

There were differences between electrophoresis bands in the fingerprint of 18s rDNA of each microalgae by double enzyme digests, and every lane was easily distinguished. Therefore, this method could be used to analyze different categories (Figure 2).

After analysis of each restriction map, a matrix analysis could be done with the Ntrys software. The evolutionary tree showed that all the microalgae could be divided into 11 different groups (Figure 3), and 2-3 strains of each group were selected randomly for 18s rDNA molecular identification.

In order to identify microalgae species suitable for lipid production and their environmental distribution, RFLP method combined with microalgae 18s rDNA sequence analysis was employed to determine all strains' taxonomy position. The results of molecular identification showed that all of the 52 strains belong to 11 different genera (Table 2).

DISCUSSION

The aim of this experiment is to realize an extensive study on biodiversity of microalgae strains suitable for lipid production through isolation, identification, classification of the wide set of microorganisms and the determination of their lipid content. The traditional microbial classification and identification methods are based on the microorganisms phenotype characteristics, such as growth, morphology, nutrition, physiological and biochemical characteristics etc. However, these methods are very time-consuming, tedious and easily influenced by operation approach. In recent years, with the development of molecular biology technology, more and more applications such as RFLP, random amplified polymorphic DNA (RAPD), enterbacterial repetitive intergenic consensus-PCR (ERIC-PCR) have been introduced in the research fields of the microbial population structure, system diversity and dynamic change. These techniques overcome the limitations of traditional methods, and are playing a greater role in the development and advances of the microbial classification, identification and diversity analysis.

Validation of RFLP method

The analysis of RFLP method depends largely on how to choose the ideal nucleic acid enzymes. We used double enzyme *Hha* I and *Hae* III to digest the 18s rDNA fragments in this experiment. Combining the enzyme cut map and sequence alignment results, it was found that the above two enzymes could be used to correctly distinguish different microalgae, and their genus taxonomic position.

Through the 18s rDNA Molecular Identification approach, we found that among all 34 strains the microalgae No.17, 22, 26 belonged to the same Genus *Scenedesmus*, and No.2, 8, 28 were different from the above four in taxonomic position. After the analysis of the enzyme cut map, it was found that among the 18s rDNA some of the digesting products of microalgae No.17, 22, 26, 34 were very similar, and others were significantly different. The results had been confirmed by repeated trials. Thus we could infer that the classification of different microalgae could effectively be done through restriction fragment length polymorphism (RFLP) analysis with the extinction enzymes *Hha* I and *Hae* III; and the taxonomic position can be accurate to Genus. The results show that the method of RFLP is simple and relatively

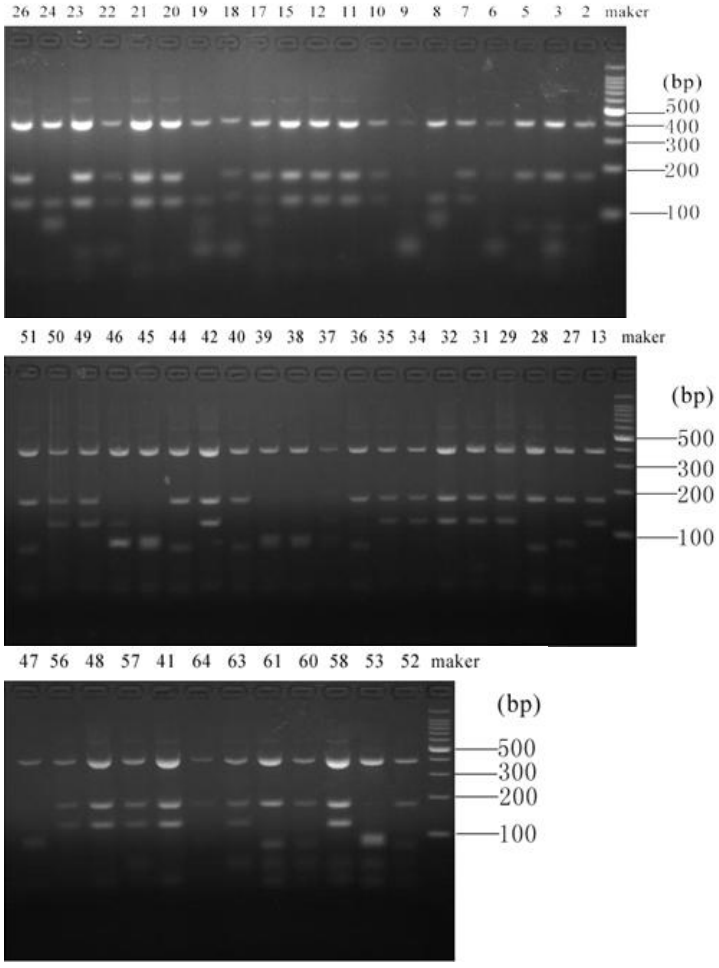

Figure 2. The electrophoresis maps of the fragments of 18srDNA by double enzyme digest.

cheap in species identification (Thaenkham et al., 2011).

Biodiversity of oleaginous microalgae

The lipid contents of all microalgae were quite different in Northern Qinghai-Tibet Plateau. The range of the lipid contents were between 3.26 to 25.47% (Figure 4). The results show that the oleaginous microalgae were extremely rich in the Northern Qinghai-Tibet Plateau. Microalgae of high quality could be separated from these special habitats, which could provide efficient and more economical microalgae species for industrial production of microalgae grease.

The results of molecular identification showed that all of the 52 strains belonged to 11 different Genera. 22 strains of them belonged to *Scenedesmus* Genus accounted for 42.31%, and other strains belong to *Chlorella*, *Nannochloris*, *Dictyosphaerium*, *Ankistrodesmus*, *Micractinium*, *Botryococcus*, *Pseudomuriella*, *Muriella*, *Chlamydomonas*, and*Graesiella*, respectively (Figure 5). Thus, it could be seen that the oleaginous microalgae in the Northern Qinghai-Tibet Plateau special habitat showed extremely rich diversity.

Relationship between habitat and distribution of the oleaginous microalgae

The lipid contents of microalgae were very different because of their different growth environments. The numbers of microalgae whose lipid contents were higher than 15% (including 15%) isolated from water and soil samples were 18 and 15, respectively. Obviously, the amount and species of microalgae in water samples were richer than those in soil samples (Figure 6).

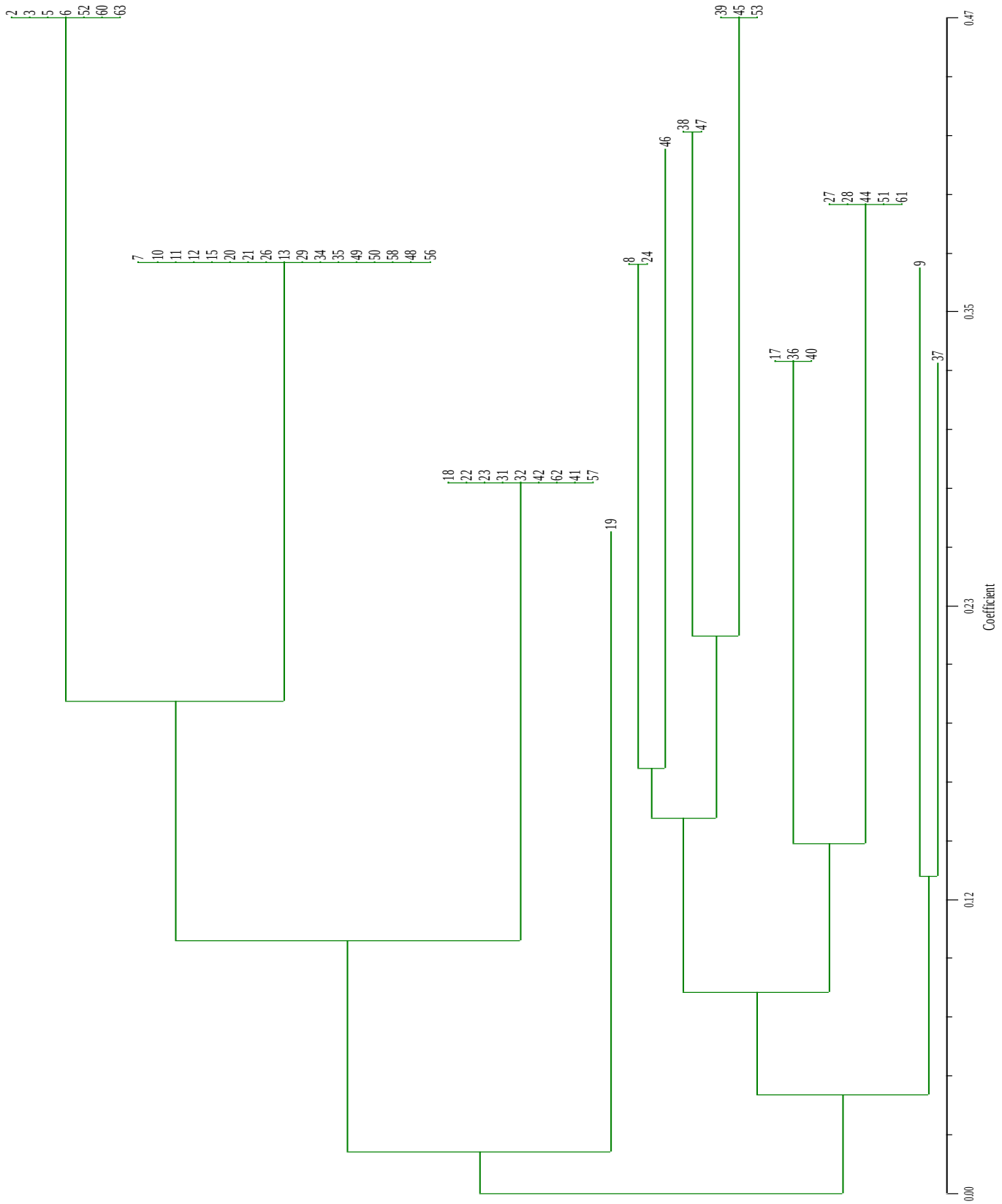

Figure 3. Classification cladogram of 52 microalgae constructed with Ntrys software

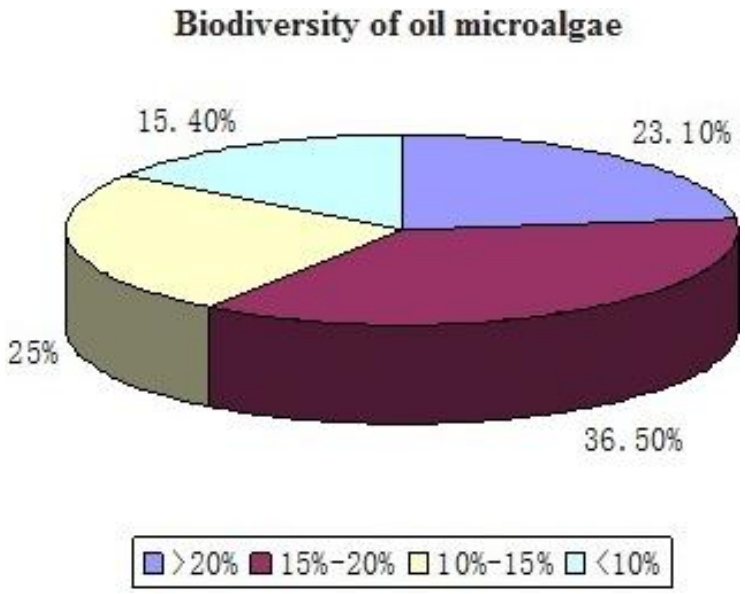

Figure 4. Biodiversity of lipid content in microalgae.

Table 2. The results of molecular identification.

Strain no.	Name	Strain no.	Name
2	*Chlorella* sp.	34	*Scenedesmus regularis*
3	*Chlorella* sp.	35	*Scenedesmus regularis*
5	*Chlorella* sp.	36	*Dictyosphaerium* sp.
6	*Chlorella* sp.	37	*Botryococcus* sp.
7	*Scenedesmus communis*	38	*Nannochloris* sp.
8	*Ankistrodesmus gracilis*	39	*Nannochloris* sp.
9	*Pseudomuriella aurantiaca*	40	*Dictyosphaerium* sp.
10	*Micractinium* sp.	41	*Scenedesmus* sp.
11	*Scenedesmus pectinatus*	42	*Graesiella emersonii*
12	*Scenedesmus deserticol*	44	*Dictyosphaerium* sp.
13	*Scenedesmus regularis*	45	*Nannochloris* sp.
15	*Scenedesmus* sp.	46	*Muriellaterrestris*
17	*Scenedesmus* sp.	47	*Nannochloris* sp.
18	*Scenedesmus subspicatus*	48	*Scenedesmus regularis*
19	*Chlamydomonas* sp.	49	*Scenedesmus regularis*
20	*Scenedesmus* sp.	50	*Scenedesmus regularis*
21	*Scenedesmus* sp.	51	*Dictyosphaerium* sp.
22	*Scenedesmus subspicatus*	52	*Chlorella sorokiniana*
23	*Scenedesmus subspicatus*	53	*Nannochloris* sp.
24	*Ankistrodesmus* sp.	56	*Scenedesmus* sp.
26	*Scenedesmus* sp.	57	*Scenedesmus* sp.
27	*Dictyosphaerium* sp.	58	*Scenedesmus regularis*
28	*Dictyosphaerium tetrachotomum*	60	*Chlorella sorokiniana*
29	*Scenedesmus regularis*	61	*Pseudomuriella aurantiaca*
31	*Graesiella* sp.	62	*Scenedesmus* sp.
32	*Graesiella* sp.	63	*Chlorella sorokiniana*

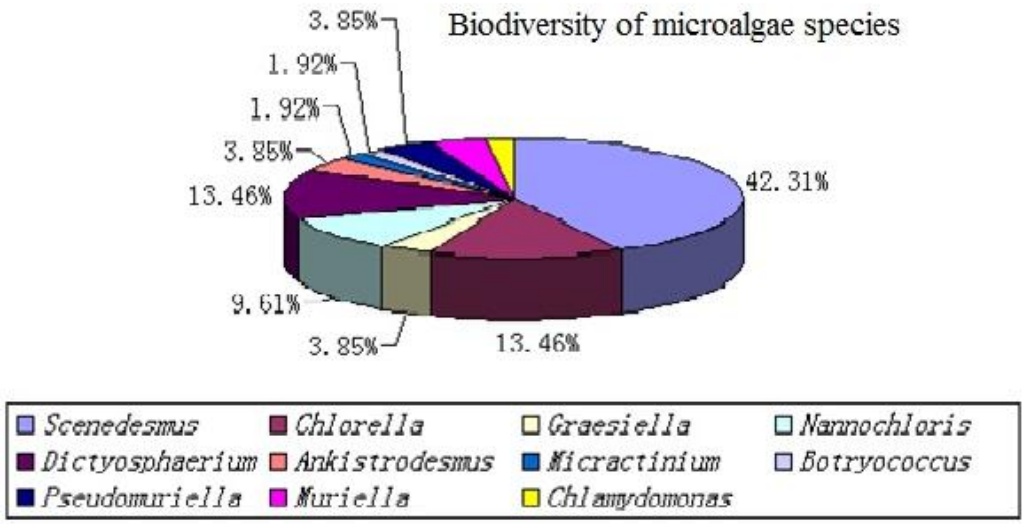

Figure 5. Biodiversity of microalgae species.

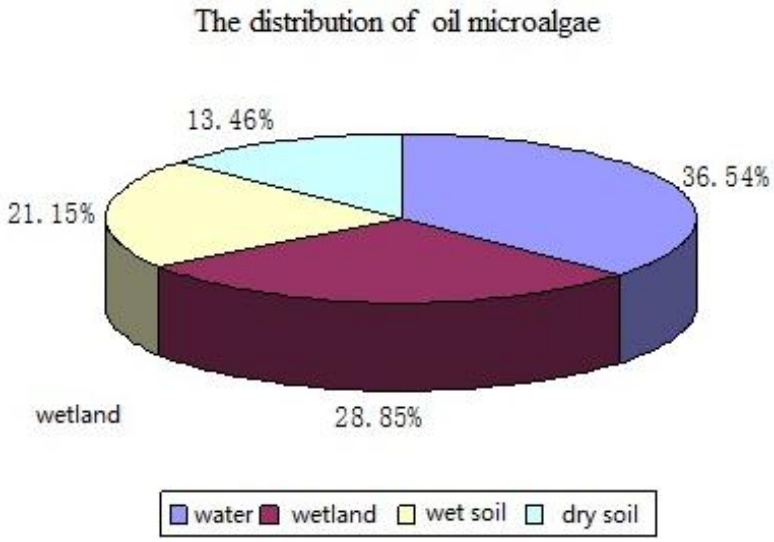

Figure 6. The distribution of oleaginous microalgae.

Our experiment results showed that it was easier to find more microalgae with high lipid content in water and wet soft soil (such as wetland, grass, hot spring mouth) than in the permafrost, hard court and sand hill soil samples. In addition, the last demonstrated lower average lipid content.

For example, in *Scenedesmus*, there were 13 strains isolated from the water, of which 10 strains' lipid contents were higher than 15% (including 15%), and there was the highest strain with the lipid content of 25.47%. However, there were only nine strains isolated from the soil samples, and there were only five strains of microalgae whose lipid contents were higher than 15% (including 15%); the highest one was 22.02%. Furthermore, among the microalgae separated from the soil samples, No. 51 and 58 had higher lipid contents than others separated from the river mouth grass and hot springs silt, respectively.

Obviously, microalgae with high lipid contents are easier to grow in the water environment. Therefore, we can infer that, moist environment is more suitable for the isolation of oleaginous microalgae.

Finding new energy resources to replace petroleum has

been a hot topic worldwide since the energy crisis. Microbial lipid is a new resource after animal fats and plant oils because it has many advantages over the conventional energy resources. Although, at present, the research on oleaginous microorganism focuses on exploring oleaginous microorganisms by screening the existing microorganisms or optimizing the microbial fermentation conditions to improve the lipid content; yet there are few researches for separation and diversity of the oleaginous microorganisms in different environments, which has limited the oleaginous microorganisms species. This experiment is mainly to study oleaginous microalgae biodiversity in Northern Qinghai-Tibet Plateau in order to provide the basis and cheap raw materials for the industrial production of microalgae lipid. We believe that, with the growing concern on microalgae lipid and the ceaseless improvement of technology and method, microalgae lipid will become a new direction of the lipid industry development.

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African Journal of Microbiology Research

Full Length Research Paper

Efficient extraction and rapid quantitative determination of nucleoside compounds from *Cordyceps jiangxiensis*, a new *Cordyceps* producing-cordycepin

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***Cordyceps*, a well-known and precious traditional Chinese medicine, has received increasing attention worldwide due to its outstanding curative effects for different diseases. Nucleosides are the main active compounds of *Cordyceps*, and are usually the chemical marker used for the quality control of *Cordyceps* and its bioproduct. In this study, an optimal condition for extracting nucleosides in *Cordyceps jiangxiensis* was achieved by an orthogonal design as follows: 15% ethanol-water extraction solvent, 10 min extraction time, 20:1 solvent to sample ratio, and 1 extraction frequency. Also, a simple, rapid, and reliable method by High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) was successfully used to simultaneously and qualitatively identify seven nucleosides compounds in *C. jiangxiensis*. Determination was achieved on a Shimadzu VP-ODS column (4.6 × 250 mm i.d. 5 µm) using a gradient elution with a methanol/water mobile phase. All calibration curves showed good linearity (R^2>0.99) within the test ranges. The overall relative standard deviations for intra- and inter-day of seven analytes were less than 4.2%. Under the developed method, the findings indicated that uridine was the most abundant nucleoside, adenosine was inferior to uridine, and cordycepin with antitumor activity was also detected in *C. jiangxiensis*. The method developed might be applied as an alternative approach in assessing the quality of other *Cordyceps* species.**

Key words: *Cordyceps jiangxiensis*, nucleosides, extraction, quantitative detection, High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD).

INTRODUCTION

Cordyceps, an entomopathogenic fungus, has received increasing attention worldwide in the past decades due to its medicinal values for curing various diseases in traditional Chinese medicine (Zhong and Xiao, 2009; Xiao et al., 2009a; Liang, 2007). *Cordyceps jiangxiensis*, a medicinal macrofungus native to eastern China, has been collected and identified as a new species of the genus *Cordyceps* several years ago (Liang, 2007; Xiao et al., 2006a). Furthermore, it has been used as a folk recipe of Chinese medicine for centuries. Its pharmacological properties present highly promising prospects for the development of nutraceuticals and new drugs, and have attracted much attention in China (Liang, 2007). However, the natural sources of *Cordyceps* are very limited because of their host specificity and confined environmental requirements for growth, which are also the technology bottleneck for industrialization. Fortunately, cultured *Cordyceps* mycelia possess the same bioactive

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ingredients and similar pharmacological effects as natural *Cordyceps* (Zhong and Xiao, 2009; Xiao and Zhong, 2007). The large-scale production of *Cordyceps* by submerged fermentation has proven to be a promising method to meet human needs and to reduce the pressure on natural sources (Zhong and Xiao, 2009; Xiao and Zhong, 2007). Accordingly, proper conditions for the culture of *C. jiangxiensis* in submerged fermentation to produce mycelia and intra-/exocellular polysaccharides have been developed (Xiao et al., 2004, 2006). Recently, polysaccharides or extracts of *C. jiangxiensis* have been confirmed to possess potent anti-tumor pharmacological properties through the apoptotic pathway of caspase activation (Xiao et al., 2006b, Xiao and Zhong, 2008), and scavenging free-radical activity (Xiao et al., 2011a). Its chemical composition demonstrates that it is a valuable and promising source for the development of healthy foods and drugs (Xiao et al., 2009b, 2011b, 2012). However, limited information is available about the quality control for *C. jiangxiensis* and its bioproduct.

Nucleosides play important roles in the regulation of various physiological processes in body (Gill and Indyk, 2007). Moreover, some nucleosides and their analogs such as 5-fluorouracil, 2'3'-dideoxyadenosine, acyclo-guanosine, have been used as antitumor or antiviral agents (Holliday and Cleaver, 2008; Klubes and Cerna, 1983; Clercq and Field, 2006). 3'-deoxyadenosine (cordycepin) from some *Cordyceps* species such as *Cordyceps militaris, Cordyceps kyushuensis* shows potent antitumor and antimicrobial activities (Sun et al., 2003; Li et al., 2006; Xiao and Zhong, 2007). N^6-(2-hydroxyethyl) -adenosine first separated from *Cordyceps* pruinosa is an efficient calcium antagonist (Liang, 2007). Though the efficacy of *Cordyceps* may not be completely derived from nucleoside compounds, nucleosides have been recognized as major active components in *Cordyceps* (Li et al., 2006; ECPC, 2005; Gong et al., 2004; Holliday and Cleaver, 2008). According to previous reports (Yang et al., 2007; Xiao et al., 2009b; Xiao et al., 2013), approximate 20 nucleosides have been isolated from *Cordyceps*. Also, there are signi-ficant differences in the variety and content of nucleosides among the different *Cordyceps* species (Li et al., 2006; Yang et al., 2007; Xiao et al., 2013). The determination of nucleosides, therefore, is very important for pharmaco-logical study and quality control of *C. jiangxiensis*.

In recent years, the simultaneous determination of multiple components, also frequently considered as quality control standards, have become the trend in the quantitative analysis of Chinese medicine. For example, chromatographic fingerprinting of nucleosides is one of the quality control standards for *Cordyceps* products (Li et al., 2006; Yu et al., 2007; Xiao et al., 2013), which may also be used as a marker in identifying authentic *Cordyceps* from imitations in the market (Hsu et al., 2002). To date, many methods are available for the quantification of nucleosides in *Cordyceps*, including thin layer chromatography, high performance liquid chromate-graphy (HPLC), capillary electrophoresis, liquid chromatography–mass spectrometry, and ion-pair reverse-phase chromatography (Yang et al., 2007; Xiao et al., 2013). However, the quality control standards of *Cordyceps* still remain controversial. In an attempt to establish a reliable quality control system for *Cordyceps*, it is first necessary to select a reliable analytical method to identify the profile of nucleosides in *Cordyceps*. Based on significant differences of nucleosides among the different *Cordyceps* species, further, for certain *Cordyceps* species, selecting major and specific nucleosides for quality control is also necessary. Among the above methods mentioned, HPLC might be the most available determiner of nucleosides in *Cordyceps* products because of its specificity, sensitivity, convenience, economy, and availability (Yu et al., 2007; Yang et al., 2007; Li et al., 2004; Xiao et al., 2013). However, the separation of the different nucleosides and their analogs is very difficult during *Cordyceps* preparations because of the similarities in their chemical structures. Determining high-efficiency HPLC conditions for the simultaneous separation and determination of nucleosides with good resolution for adjacent peaks within a short analysis time is essential. For wavelength detection, diode array detection (DAD), with the additional UV-vis spectral information, allows for easy qualitative analysis of peaks in a fingerprint chromatogram compared with common UV detectors. DAD can also record a series of chromatograms along a wide range of wavelengths, which allows fast and simple wavelength optimization (Liang et al., 2009; Xiao et al., 2013).

In the present study, HPLC-DAD detection of seven major nucleoside compounds in *C. jiangxiensis* (uracil, uridine, inosine, guanosine, adenine, adenosine, and cordycepin) was conducted for their simultaneous separation and determination, after a simple ultrasonic extraction procedure. It is the first to develop a method for the simultaneous determination of the seven main nucleoside compounds in *C. jiangxiensis*, which is important in pharmacological studies and quality control of *Cordyceps* products.

MATERIALS AND METHODS

Chemicals

Standards of uracil, adenine, uridine, inosine, guanosine, adenosine, and cordycepin were purchased from Sigma (St. Louis, MO, USA). LC-grade methanol was purchased from Tedia Company, Inc. (OH, USA). Ultrapure water was prepared using a Millipore Milli-Q Plus system (Millipore, Bedford, MA, USA). The other reagents were of analytical grade.

Sample

The voucher specimens of *C. jiangxiensis* were deposited at the Institute of Fungal Resource, University of Guizhou, Guiyang, China. As previously described (Liang, 2007; Xiao et al., 2004,

Table 1. Factors and levels of nucleosides extraction for the $L_9(3^4)$ orthogonal design.

Factors level	A	B (min)	C (v/m)	D(times)
1	ultrapure water	30	20:1	2
2	5% methanol water-solution	20	10:1	3
3	15% methanol water-solution	10	30:1	1

A, B, C, and D represent correlation factors of nucleoside extraction: extraction solvent, extraction time, solvent to sample ratio, and frequency, respectively. Numbers 1-3 in the first column represent levels of each factor in the orthogonal design.

Table 2. $L_9(3^4)$ orthogonal test results of nucleosides extraction and its analysis for *Cordyceps jiangxiensis* ($\bar{x}$±s.d., n=3).

Runs	A	B	C	D	Total peak areas of nucleosides tested
1[#]	1[†]	1	1	1	5950251[‡]
2	1	2	2	2	2639998
3	1	3	3	3	4421425
4	2	1	2	3	4550169
5	2	2	3	1	2102561
6	2	3	1	2	4468190
7	3	1	3	2	3607988
8	3	2	1	3	6829044
9	3	3	2	1	5300164
K1[§]	13011674	14108408	17247485	13352976	
K2	11120920	11571603	12490331	10716176	
K3	15737196	14189779	10131974	15800638	
R[*]	4616276	2618176	7115511	5084462	
optimal level	3	3	1	3	

[†] The arrangement of columns A-D was decided by orthogonal design for $L_9(3^4)$, and A, B, C, and D represents extraction time, temperature, extractant, and frequency, respectively. [#] Each row of the experimental run number represents one experimental replicate, and each run was replicated thrice. [‡]Values are mean of triple determinations. [§] K_i^X the peak area of nucleosides tested in *Cordyceps jiangxiensis* in thrice experiment at X_i. [*]Symbol *R* means the maximum value of K_i^X minus the minimum value of its. symbol *X* in the above represents A, B, C, and D, respectively, and symbol *i* represents each level.

2006a), *C. jiangxiensis* was prepared through liquid fermentation technology. Previous studies have indicated that the extraction of nucleosides from *C. sinensis* and its substitutes using water is the best selection method for the determination of nucleosides (Yang and Li, 2008). Thus, water was used as extractant for the extraction of nucleosides in the preliminary experiment. Up to 1.0 g of dried powder *Cordyceps* (60-100 mesh) was mixed with 10 mL ultrapure water, and then extracted at room temperature for 30 min using an ultrasonic processor. The procedure was repeated twice. After the combined extract was centrifuged at 4500 g for 15 min, the supernatant (extract sample) was stored at 4°C in a refrigerator before HPLC analysis.

Orthogonal design and procedure for sample preparation

Based on previous reports (Yang and Li, 2008) and consistence with our preliminary experimental results, ultrasonic extraction was more suitable for the preparation of nucleosides compounds of *Cordyceps*, not microwave-assisted extraction or hot-water extraction. The extraction conditions were further optimized by $L_9(3^4)$ orthogonal design (Zhao, 2006). In this study, extraction solvent, extraction time, solvent to sample ratio, and frequency were the orthogonal factors for the extraction of nucleosides from the *Cordyceps* samples. The design factors and the levels of orthogonal layout are shown in Table 1. The experimental conditions of each experimental run are listed in Table 2, including the experimental results in the last column. For the extraction treatment, 0.4 g of sample was placed in a 50 mL disposable centrifuge tube, and mixed with a determined volume of extraction solvent for each of the experimental runs. Thereafter, the mixture was allowed to stay overnight at 4ºC, and then it was placed in an ultrasonic water bath to be treated at room temperature according to the orthogonal design conditions. After extraction, the tubes were centrifuged at 5000 rpm for 10 min. The extract was collected, its total volume measured, and then it was stored at 4ºC for future use. Each experimental run was repeated three times.

Preparation of standard solutions

The nucleosides were dissolved in ultrapure water until a 1.0 mg/mL solution was achieved. A certain amount of the stock solution was transferred to a 10 mL volumetric flask, and filled up to its volume with the same solvent to obtain the desired concentration. All solutions were found stable when stored at 4°C for three weeks. The extract sample was then transferred to a 25 mL volumetric flask, filled up to its volume with the extraction solvent, and filtered through a 0.22 µm Millipore filter prior to injection into the HPLC system.

HPLC analysis

Chromatographic analysis was performed using a Shimadzu Series LC-20A HPLC (Shimadzu Kyoto, Japan), equipped with a binary high-pressure pump, a CTO-10Avp column oven, and a photodiode array detector connected to an LCsolution Software. The column used for separation was the Shimadzu VP-ODS column (4.6 × 250 mm i.d. 5 µm) and the Shimadzu VP-ODS C18 guard column (4.6 × 12.5 mm). As previously described (Yu et al., 2007; Hsu et al., 2002; Li et al., 2004; Yang and Li, 2008), phosphate buffer, acetate, and acetonitrile have often been used as the mobile phases for the HPLC analysis of nucleosides. The optimized chromatographic conditions involving two mobile-phases systems with ultrapure water (W) and methanol (M), as well as elution conditions, were determined. The elution conditions were as follows: 0 min to 3.0 min, isocratic 15% M; 3.0 min to 3.5 min, linear gradient 15% to 24% M; 3.5 min to 8.5 min, isocratic 24% M; 8.5 min to 9.0 min, linear gradient 24 to 35% M; 9.0 min to 15.0 min, isocratic 35% M. Finally, the column was washed with 100% B for 10 min before reconditioning the steps of the column using 15% M isocratic for 15 min. The flow-rate was 1 mL/min and the injection volume was 20 µL. All injections were repeated three times to ensure reproducibility. The system was operated at 35°C. The detecting wavelength of the photodiode array detector was set to the range of 190 to 500 nm, and the nucleosides were monitored and quantified at 260 nm. The nucleosides were identified by comparing their retention time, purity coefficient, and spectrum against known standards. The external standard method was used to determine the nucleosides.

RESULTS AND DISCUSSION

Screening of conditions for the separation and determination of different nucleosides

In the present study, an optimized strategy for HPLC conditions was performed on the extract sample of *C. jiangxiensis*. Based on the absorption maxima of seven analytes and the stable baseline of the UV spectra with 3D chromatograms of HPLC-DAD detection, a 260 nm detection wavelength was used. The results suggest that the separation was improved when the column temperature was raised to 35°C, and the mobile phase was delivered at a flow rate of 1.0 mL/min.

For the mobile phase system, the results showthat these compounds cannot be separated by an isocratic elution system, such as acetonitrile, methanol, or buffer solution (water-KH_2PO_4), or by two mobile-phase isocratic elution system, such as acetonitrile/water, acetonitrile/buffer solution, methanol/water, and methanol/buffer solution, because of their similar chemical structures, as shown in Figure 1. Thus, the above four double mobile-phase systems with linear gradient elution condition (A: 0% to 15%; B: 100% to 85%) were further used for their separation. Most of the compounds were separated within 35 min, except for inosine, guanosine, and adenine, in which the analytical duration of the methanol/water system was less than 20 min (Figure 2A). Furthermore, taking into account the causticity of the salt solution in the HPLC system and the high cost of acetonitrile, the methanol/water system was used for further study. For the effective separation of each component of interest from the sample matrix, complex gradient HPLC systems are usually required (Yang et al., 2007; Liang et al., 2009). Thus, to obtain the desired separation effects for these compounds, the methanol/water system, with alternating isocratic and gradient elution strategies, was used in this study. Relatively good peak profiles and resolutions for inosine, guanosine, and adenine were obtained by prolonging the initial elution time from 0 min to 3 min (Figure 2B, C). The initial concentrations of the mobile phase increased from 8 to 15%, then from 15 to 24%, resulting in the desired separation profile with higher peak profile and resolution, and shorter analytical time of about 12 min, as shown in Figure 2D. As a result, the final elution conditions used for the methanol (M)/water (W) mobile phase were as follows: 0 to 3.0 min, isocratic 15% M; 3.0 to 3.5 min, linear gradient 15 to 24% M; 3.5 to 8.5 min, isocratic 24% M; 8.5 to 9.0 min, linear gradient 24 to 35% M; 9.0 to 15.0 min, isocratic 35% M. The analytical time was much less than those in similar reports (Yu et al., 2006, 2007).

Optimal extraction conditions for nucleosides in cultured *C. jiangxiensis*

High-efficiency extraction is necessary for the quantitative determination of specific compounds in *Cordyceps* products before analysis. To obtain a relatively high yield of the total investigated nucleosides, an optimized extraction procedure of nucleosides in *C. jiangxiensis* was developed using a $L_9(3^4)$ orthogonal layout (Tables 1 and 2). The peak areas were used as tested indices in this study. Among the nine experimental groups, the highest peak area of the total investigated nucleosides in *C. jiangxiensis* reached 6,829,044, as shown in Table 2. The eighth group (A3B2C1D3) obtained the highest peak area, whose levels of homologous factors involved 15% ethanol water-solution extraction solvent, 20 min extraction time, 20:1 solvent to sample ratio, and one extraction frequency. The fifth group obtained the lowest total peak areas of nucleosides with 2,102,561, whose corresponding factors and levels were A2B2C3D1, including 5% ethanol water-solution extraction solvent, 20 min extraction time, 30:1 solvent to sample ratio, and two extraction frequencies.

As to the effect of the extraction factors and levels on the extraction yield of nucleosides in *C. jiangxiensis*, the degree among the factors was C>D>B>A (extraction time > extractant > extraction frequency > extraction temperature), according to the order of magnitude of the R values (maximum difference) (Table 2). This order was also demonstrated by the F and/or P value in the variance analysis in Table 3. Among the four factors, only extraction time (B) was not associated with the extraction yield of nucleosides in *C. jiangxiensis* (Table 3). The other three factors have extremely significant effect, with $p<0.01$. Based on the maximum K value (the gross extraction yield of nucleosides in three instances) of each

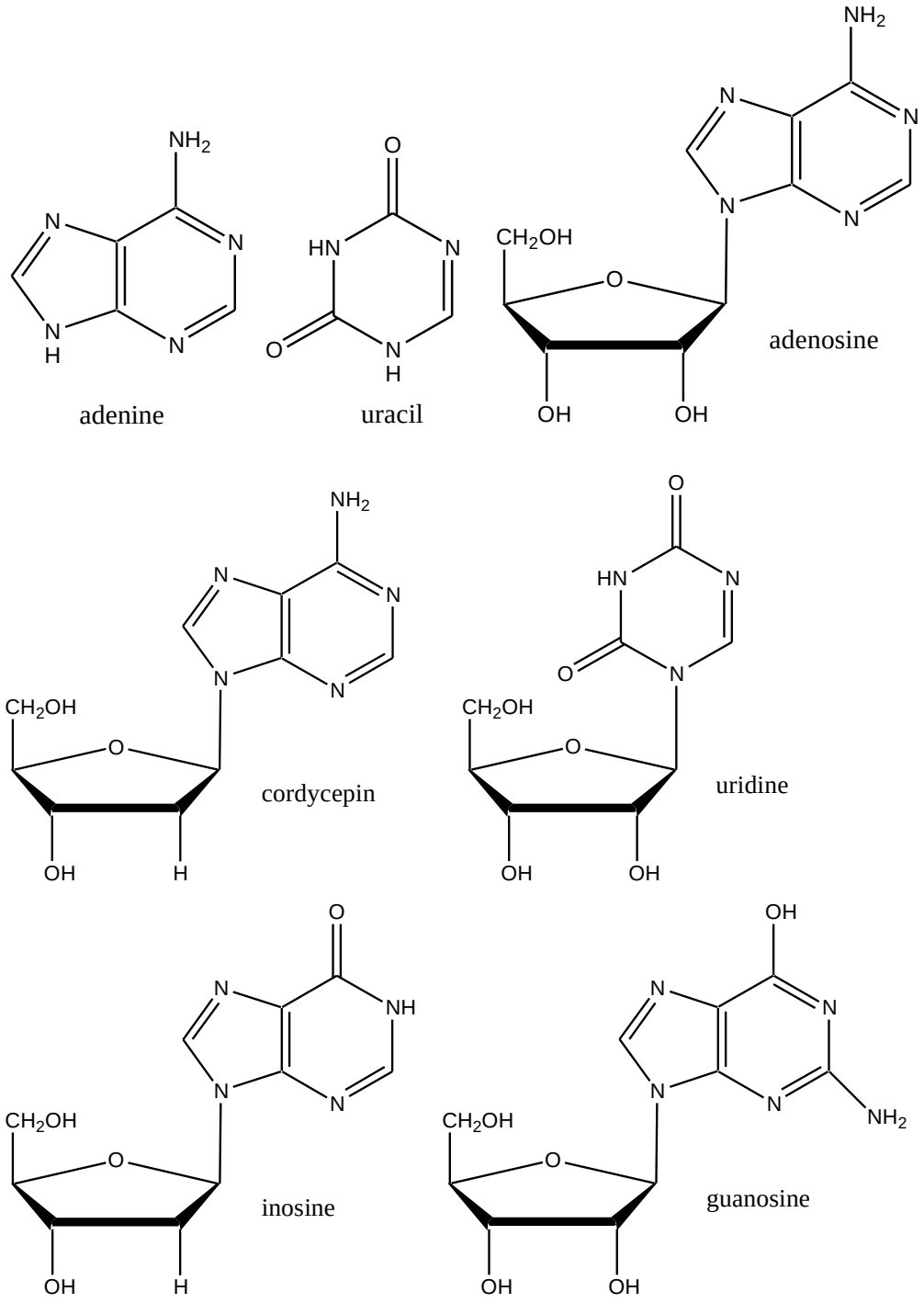

Figure 1. Chemical structures of nucleosides and nucleobases.

column in Table 2, the optimal level of each factor for extracting nucleosides in *C. jiangxiensis* (A3B3C1D3) was confirmed. The optimal condition for extracting nucleosides in *C. jiangxiensis* are as follows: 15% ethanol water-solution extraction solvent, 10 min extraction time, 20:1 solvent to sample ratio, and 1 extraction frequency.

Validation procedure

The stock standard of nucleosides was prepared using 1 mg/mL solution. Additional calibration levels were prepared by serial gradient dilution with ultrapure water. A standard calibration curve was created using these nucleo-

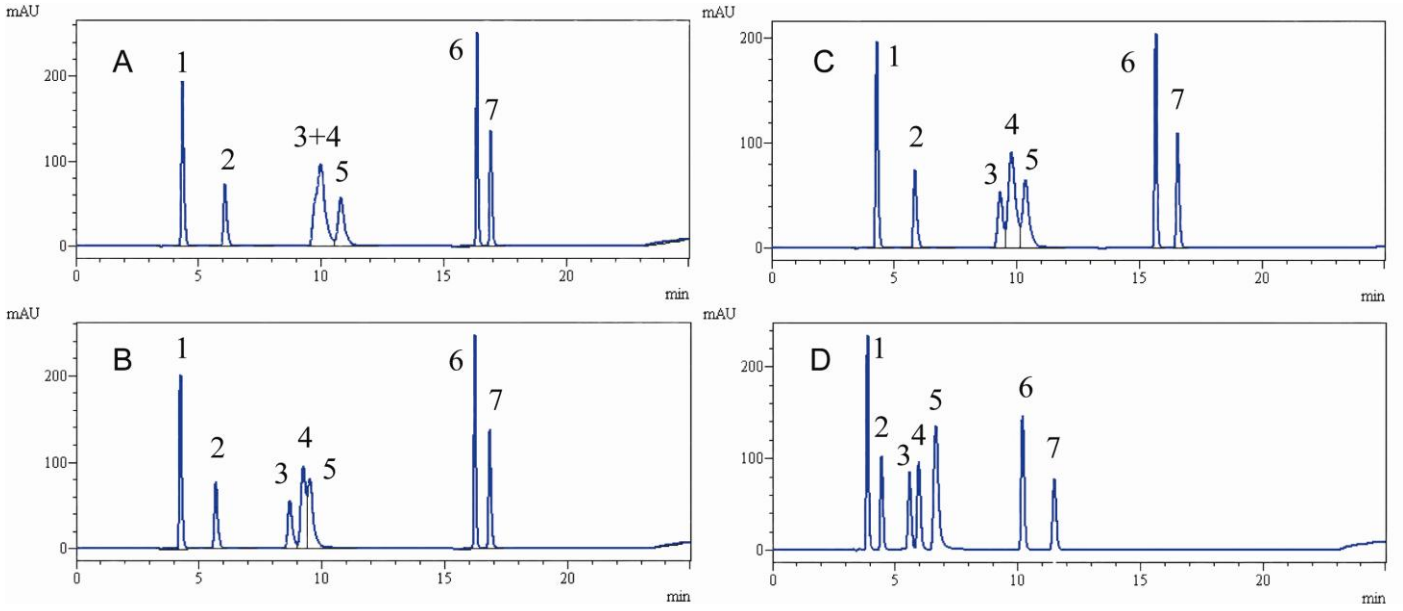

Figure 2. Chromatograms of mixed standard water solutions in different mobile phases. **A**. The elution condition for the mobile-phases, methanol (M) (0 to 15% within 20 min) and ultrapure water (W) (100 to 85% within 20 min). **B**. The elution condition for the M/W mobile-phases, 0 to 1.0 min, isocratic 8% M; 1.0 to 3.5 min, linear gradient 8 to 15% M; 3.5 to 8.5 min, isocratic 15% M; 8.5 to 9.0 min, linear gradient 15 to 35% M; 9.0 to 15.0 min, isocratic 35% M. **C**. The elution condition for the M/W mobile-phases, 0 to 2.0 min, isocratic 8% M; 2.0 to 3.5 min, linear gradient 8 to 15% M; 3.5 to 8.5 min, isocratic 15% M; 8.5 to 9.0 min, linear gradient 15 to 35% M; 9.0 to 15.0 min, isocratic 35% M; **D.** The elution condition for the M/W mobile-phases, 0 to 3.0 min, isocratic 15% M; 3.0 to 3.5 min, linear gradient 15 to 24% M; 3.5 to 8.5 min, isocratic 24% M; 8.5 to 9.0 min, linear gradient 24 to 35% M; 9.0 to 15.0 min, isocratic 35% M .**1,** uracil; **2,** uridine; **3,** inosine; **4,** adenine; **5,** guanosine; **6,** adenosine;**7:,**cordycepin.

Table 3. Variance analysis of the $L_9(3^4)$ orthogonal test results on nucleosides extraction for *Cordyceps jiangxiensis*[§].

Variance Source	Sum of Squares	Degree of Freedom	Mean Square	*F* value	Significance Level
Corrected model	36273032984774.440[#]	8	4534129123096.800	10.638	.001
Intercept	353244443369987.600	1	353244443369987.600	828.813	.000
A	7180764367283.100	2	3590382183641.551	8.424	.009
B	2954854213157.442	2	1477427106578.721	3.466	.077
C	17516186613900.450	2	8758093306950.220	20.549	.000
D	8621227790433.430	2	4310613895216.719	10.114	.005
Errors	3835848293516.000	9	426205365946.222		

[§]Statistically significant at 95% confidence level; [#] *R* Squared =0.904; Adjusted *R* Squared = 0.819.

Table 4. Regression curves, linearity, limit of quantification (LOQ), and limit of detection (LOD).

Analyte	Linear regression data			LOD	LOQ
	Linear range (μg/mL)	Regression equation	R^2	(μg/mL)	(μg/mL)
Uracil	0.95–92.00	Y=83653.97X+51089.60	0.9915	0.010	0.05
Adenine	0.96–96.00	Y=110193.50X+76283.66	0.9998	0.001	0.01
Uridine	0.88–175.00	Y=46749.92X-8299.70	1.0000	0.003	0.01
Inosine	0.88–175.00	Y=48799.90X-4571.77	1.0000	0.005	0.01
Guanosine	4.40–176.00	Y=57174.02X+30802.4	0.9997	0.003	0.01
Adenosine	1.00–100.00	Y=68802.19X-16891.63	0.9998	0.001	0.01
Cordycepin	1.13–91.00	Y=89476.15X-15045.62	0.9999	0.050	0.01

The data was present as average of three determinations. R^2, squares of correlation coefficients for the standard curves; LOD, limit of detection; LOQ, limit of quantification.

Table 5. Intra- and inter-day repeatability of the investigated analytes.

Analyte	Intra-day (n=5) R.S.D. (%)			Inter-day (n=5) R.S.D. (%)		
	H-conc. †	M-conc. ‡	L-conc. §	H-conc.	M-conc.	L-conc.
Uracil	0.65	1.43	1.30	0.48	1.00	3.99
Adenine	1.09	2.49	0.76	1.31	2.61	4.16
Uridine	0.79	1.56	0.41	2.01	1.67	2.22
Inosine	1.52	0.92	0.30	0.89	0.61	1.03
Guanosine	0.58	1.20	0.65	0.50	0.70	2.12
Adenosine	0.69	0.70	0.44	0.58	0.72	0.77
Cordycepin	0.59	0.70	0.66	0.62	0.63	1.03

R.S.D. means relative standard deviations. †H-conc. represents that the concentrations of uracil, adenine, uridine, inosine, guanosine and adenosine, cordycepin were 10.23, 10.56, 12.03, 13.22, 8.68, 10.50, and 8.10µg/mL, respectively. ‡M-con, represents that the concentrations of uracil, adenine, uridine, inosine, guanosine and adenosine, cordycepin were 1.45, 1.49, 2.64, 2.75, 2.18, 2.31, and 1.77 µg/mL, respectively. §L-conc. represented that the concentrations of uracil, adenine, uridine, inosine, guanosine and adenosine, cordycepin were 0.25, 0.29, 0.68, 0.64, 0.88, 0.67, and 0.49 µg/mL, respectively.

side water-methanol solutions at a concentration range of 0.5 to 200 µg/mL. A linear regression analysis was performed by plotting the peak areas against concentrations of nucleosides. The regression equations obtained by the least square method are listed in Table 4. Using the determined chromatographic condition, the values of the correlation coefficient R^2 of the calibration curves of the seven analyses were all more than 0.99. These results suggest good relationship between the nucleoside concentrations and their peak areas within the tested range.

The limit of detection (LOD) and the limit of quantification (LOQ) were determined, using the chromatographic conditions previously developed, based on the signal-to-noise ratios (S/N) of 3:1 and 10:1. The lowest concentration of the working solution containing seven reference analytes were diluted with ultrapure water to desired concentrations, and the aliquots were injected into the HPLC for analysis. In this study, five replicates of blank samples were analyzed. Three and ten average standard deviations of the blank responses to the corresponding slope of the calibration curve were regarded as LOD and LOQ, respectively. The LOD and LOQ for all tested analytes were all lower than 0.05 µg/mL, as shown in Table 4. This finding suggests that the quantification of each analyte investigated was at full capacity.

Intra- and inter-day variations were used to verify the precision of the method developed. For the intra- and inter-day variability tests, the standard solutions were determined in triplicate, five times daily, for three consecutive days. As shown in Table 5, precisions for all the analytes were calculated using the relative standard deviation (%RSD). The computed RSDs were 0.30 to 2.49% (n = 5) for the intra-day assay and 0.48 to 4.16% (n = 5) for the inter-day assay on three different concentrations. The %RSD was within the acceptable limit of 5%.

A recovery test was used to evaluate the accuracy and stability of the method. The *C. jiangxiensis* materials were spiked with known amounts of mixed standards at two concentration levels before extraction. The spiked samples were extracted with 15% ethanol water-solution following the procedure above-mentioned. Three replicates were performed for the test, and background levels were subtracted in all recovery determinations. The recovery of the investigated analytes ranged from 85.23 to 104.36%, and their %RSD values were all less than 5.5%, as shown in Table 6. The findings indicate the accuracy and reliability of the method developed. Likewise, the accuracy of the method was independent from the compound concentration and chemical structure.

In addition, selectivity was validated using an extract of *C. jiangxiensis* and a mixture of available standards optimizing separation and detection. The purity of the peaks was checked using a diode array detector through multivariate analysis. The three spectra of each peak, corresponding to the upslope, apex, and down slope, were normalized using a computer and then super-imposed. The peaks were considered pure when the three spectra coincided (match factor was ≥95%).

The above results show that the quantitative analysis of the seven investigated nucleosides from *C. jiangxiensis* materials by HPLC-DAD is satisfactory and feasible.

Application identification and quantitative determination of the investigated nucleoside compounds in *C. jiangxiensis*

The HPLC-DAD analysis presented a very detailed characterization of the investigated analytes in cultured *C. jiangxiensis* qualitatively and quantitatively. The typical HPLC chromatogram of methanol-water extracts from *C. jiangxiensis* detected at 260 nm is shown in Figure 3. Comparing the retention time and UV spectra with known standards, identification of the peaks of the investigated analytes was performed in *C. jiangxiensis* using the developed chromatographic method. The known analytes for peaks 1 to 7 were identified as uracil, uridine, inosine, adenine, guanosine, adenosine, and cordycepin, respectively, as shown in Figure 3. Based on the typical

Table 6. Recoveries from the assay of seven investigated analytes.

Analyte	Orignial (μg)	Spiked (μg)	Found (μg)	Recovery[†] (%)	R.S.D.[§] (%)
Uracil	4.30	10.21	13.94[†]	94.42	5.49
	4.45	1.47	5.95	102.04	
Adenine	1.60	10.73	12.30	99.72	2.28
	2.56	1.45	3.96	96.55	
Uridine	39.01	12.36	51.22	98.79	3.34
	40.81	2.60	43.26	94.23	
Inosine	-[#]	13.16	13.15	99.92	3.07
	-	2.75	2.87	104.36	
Guanosine	1.80	8.64	10.52	100.93	0.01
	1.88	2.18	4.05	99.54	
Adenosine	13.53	10.48	23.69	96.95	0.01
	14.14	2.29	16.36	96.09	
Cordycepin	0.85	8.09	8.05	89.00	3.06
	0.93	1.76	2.43	85.23	

[†] The data was present as average of three determinations. [†] Recovery (%) = (found amount −original amount)/ spiked amount×100. [§] R.S.D. (%) = 100× (S.D./mean). [#] Under the limit of quantitation.

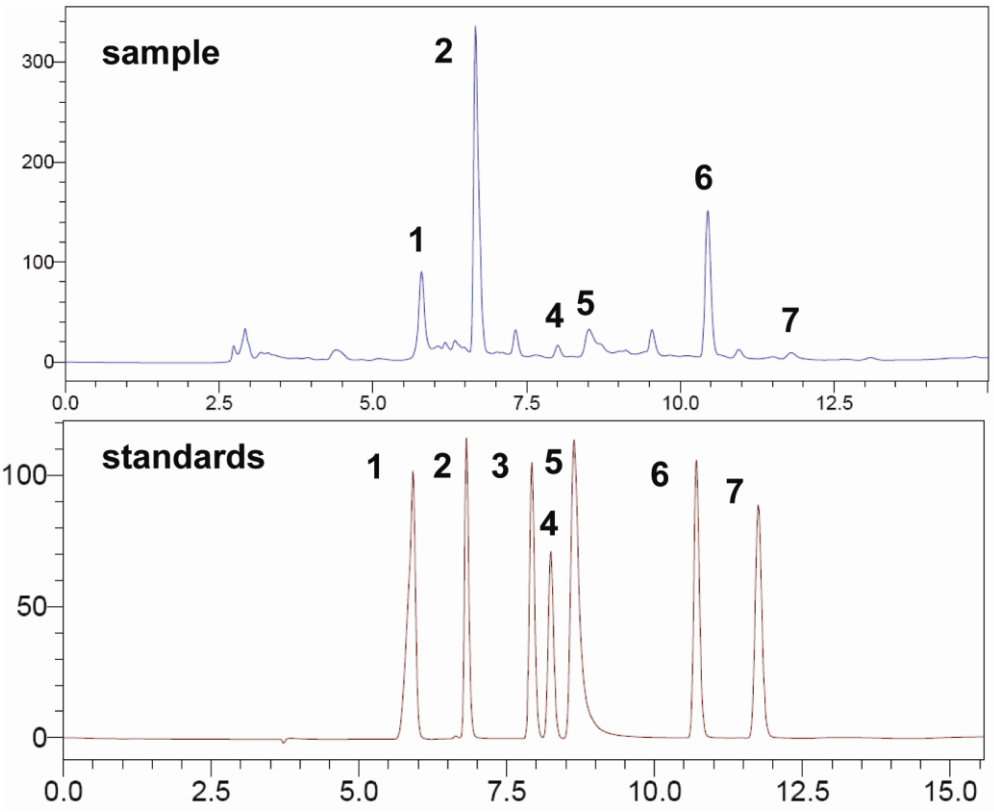

Figure 3. Typical HPLC chromatogram at 260 nm for mixed standards and cultured *C. jiangxiensis*. 1, uracil; 2, uridine; 3, inosine; 4, adenine; 5, guanosine; 6, adenosine; 7, cordycepin.

chromatogram in Figure 3, uridine and adenosine are the main nucleoside compounds in cultured *C. jiangxiensis*. Similar to cultured *C. militaris* (Yu et al., 2006), inosine (peak 3) was not detected in the cultured *C. jiangxiensis*. By contrast, natural *C. sinensis* contains rich inosine ingredient (Yu et al., 2006). Cordycepin is a considerable active component in *Cordyceps*, with various pharmacological activities, such as inhibition of protein kinase acti-

activity, enhancement of cell differentiation, anti-tumor, and anti-microbial properties (Sun et al., 2003; Xiao and Zhong, 2007). However, not all *Cordyceps* species contain cordycepin. For example, *C. militaris* and *C. kyushuensis* possess abundant amounts of cordycepin (Sun et al., 2003; Yang et al., 2007; Yu et al., 2007), whereas cordycepin is not found in many *Cordyceps* preparations, such as *Cordyceps taii*, and *Cordyceps gunnii* (Xiao et al., 2009b). Herein, trace amount of cordycepin (peak 7) was found and verified in cultured *C. jiangxiensis* by comparing the retention time and the UV spectra. Using the calibration curves of the known analytes, the analytes tested were quantitatively studied. The contents of these analytes were determined as follows: uracil (890.12 µg/g), adenine (513.48 µg/g), uridine (7802.14 µg/g), guanosine (376.14 µg/g), adenosine (2705.92 µg/g), and cordycepin (177.52 µg/g). The concentrations of uridine and adenosine were significantly higher than those of natural *C. sinensis* and cultured *C. militaris* (Li et al., 2006). Although only trace amounts of cordycepin was detected in cultured *C. jiangxiensis*, the amount was markedly higher than in natural and cultured *C. sinensis* (Li et al., 2006; Hsu et al., 2002). Currently, nucleosides are believed to be the major active components in *Cordyceps* (Hsu et al., 2002; Gong et al., 2004; ECPC, 2005; Li et al., 2004, 2006; Yu et al., 2007; Yang et al., 2007; Liang et al., 2009). Furthermore, nucleoside profiles, especially those of adenosine, cordycepin, and inosine, have been considered chemical markers for the quality control of *C. sinensis* and *C. militaris* (Li et al., 2006; Yu et al., 2006). Thus, adenosine, uridine, and cordycepin can be used for markers of quality control in *C. jiangxiensis*.

Conclusions

In the present study, a rapid HPLC-DAD method was successfully devised for the first time, which can be used to simultaneously separate and identify the nucleosides in *C. jiangxiensis* and other *Cordyceps* spp. The results show that the method developed has good separation and repeatability. Due to its high selectivity and sensitivity, HPLC-DAD is a powerful tool for the qualitative and quantitative analyses of nucleosides in Chinese medicines. HPLC-DAD is also useful in the study and quality control of *Cordyceps*. Uridine, adenosine, and cordycepin can be used as markers for the quality control of *C. jiangxiensis* bioproducts.

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Occurrence of *Salmonella spp*. in laying hens during cycles of molting

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The main objective of this study was to determine the presence of *Salmonella spp*. in 30 laying hens of the third cycle. Samples were taken from the digestive tract, cloaca, feces, and organs. The sampling of feces and cloaca was conducted first, followed by the other tissues at necropsy. Feces were taken from the floor of the cages during five weeks. Thirty (30) samples of cloaca were taken at random from five hens. The sampled organs at necropsy of 30 hens were ovary, spleen, liver, gallbladder and duodenal loop. The samples were analyzed by microbiological methods and sown on agar SB at 24 and 48 h; positive cultures were incubated at an additional 24 h and suspicious-positive samples for *Salmonella spp*. were subjected to biochemical tests. Data collected from bismuth sulfite agar (ASB) cultures were analyzed statistically by Xi squared (Xi^2) test. The results show that growth was present in two samples of feces and five from the bottom of the cage, but the differences were not significant (P> 0.05). Cloaca samples were positive in the sixth sample of a bird, but it was not different between tests (P>0.05). The percentage of bacterial growth from samples of organs in the first group was 66.66 and of 50% in the second. Biochemical tests of positive samples from feces and cloaca demonstrated the presence of *Escherichia coli* and *Edwardsiella spp*., whereas in the organs *Edwarsiella spp* was present, but not *Salmonella spp*. We conclude that it was not possible to isolate *Salmonella spp*. in feces or organs of laying hens in the third cycle of laying, suggesting that the management of the farm and laying hens were appropriate to avoid contamination from initiation to final production.

Key words: Salmonella, Moulting, laying hens.

INTRODUCTION

The genus *Salmonella* belongs to the Enterobacteriaceae family composed of enteric microorganisms which are non-spore-forming Gram-negative bacilli. Some of them are part of the intestinal normal flora, whereas others are pathogens (Gordon and Jordan, 1982). Many species of *Salmonella* have been isolated from domesticated birds; some of them, including *S. pullorum*, *S. gallinarum*, *S. arizona*, *S. anatis* and *S. typhimurium* are normally pathogenic in young birds and can be of importance as zoonosis (Gordon and Jordan, 1982).

According to the epidemiology of the disease, in birds the serotypes of *Salmonella* can be divided into three major groups. The first contains *S. pullorum* and *S. gallinarum* serotypes which produce a systemic disease limited specifically to birds. The second has *Salmonella* serotypes usually isolated from feed, the environment and birds. The above mentioned serotypes are capable of causing food poisoning but do not cause disease in the birds; its importance is related with public health. The third group comprises two serotypes: *S. typhimurium* and *S. enteritidis*, which have the characteristic of both groups previously mentioned. *Salmonella* can be easily

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transmitted from animal to animal, from animal to human and from human to human through several direct and indirect ways (Mellrog, 1997; Beard, 1997; Sockett, 1991; OPS, 1992).

A broad host range serotypes including *Salmonella typhimurium* and *Salmonella enteritidis*c an cause enteritis in a wide range of host species; these two serotypes being responsible for the majority of *Salmonella* food-borne enteritis in man. Around 30000 cases of human salmonellosis are reported in the United Kingdom each year (Wall and Ward, 1999) and are often associated with consumption of contaminated poultry meat or eggs (Humphrey, 1999).

Food borne infections are an important public health problem in the United States. For the year 2010, the Centers for Disease Control and Prevention (CDCP) estimated that all food borne infections from various viral and bacterial sources caused 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths in the U.S. (Centers for Disease Control and Prevention, 2011a,b; Morbidity and Mortality Weekly Report, June 10, 2011; NCHS, 2011).

Approximately, 150 serotypes travel more or less constantly between the animal reservoir, the environment, food and man, starting from *Salmonella* ser. *typhimurium*. Some serotypes, however, have a particular preference for some animal species: Enteritidis, Hadar, Heidelberg, Saintpaul, Virchow, Senftenberg, Infantis and Kottbus and their find main distribution channel are in chickens, turkeys and ducks; Dublin and Bovismorbificans mainly infect cattle, while the Derby, Brandenburg and Panama serotypes frequently circulate among pigs. However, with the exception of very young chicks, *S*. *enteritidis* and *S*. *typhimurium* rarely cause clinical disease, but can colonize the gut of poultry (Weill, 2009).

Salmonellae may then be shed in the faeces and can lead to horizontal transmission to other birds in the flock by the fecal ± oral route and to contamination of meat by faeces at the time of slaughter. *S*. *enteritidis* may also colonize the reproductive tract, leading to the contamination of eggs (Humphrey, 1999).

For the other *Salmonella* sources included, the model estimated that around 65 (95% CI: 63-67), 28 (95% CI: 27-30) and 4.5% (95% CI: 4-5) of the estimated number of human salmonellosis cases could be attributed to laying hens (eggs), pigs and turkeys, respectively (EFSA-Journal, 2011).

The typhoidal *Salmonella* serovars are host-restricted human pathogens and include *Salmonella enteric* serotype Typhi and S. paratyphi (Uzzau et al. 2000). Non-typhoidal *Salmonella* (NTS) are zoonotic agents and a wide variety of animals have been identified as reservoirs (Mead, 1999; Adak et al., 2002; Hald et al., 2007).

In a study comparing six methods of detection of chicken infection by *S. typhimurium*, it was concluded that micro-agglutination detected the higher number of infected birds, in addition to the fact that it was also the most direct method. Isolation from cloaca was inadequate to determine a previous exposure (Williams and Whitemore, 1976). Other report found that under particular experimental conditions, isolation from chicken beds was more effective than serology to detect *Salmonella* (Williams and Benson 1978).

Some reports agree that *S. infantis* and *S. typhimurium* did not persist as much in accumulated chicken bed, as in fresh chicken bed, suggesting that the accumulated bed has an inhibitory effect on *Salmonella*. Sauter et al. (1979), studying the effect of pH over of *Salmonella* penetration on the egg shell, and their concluded was that the maximum penetration was 42% in eggs exposed to pH 7.0, when exposed to *S. typhimurium*, *S. derby* and *S. saintpaul*.

In a study on the role of organic acids and other substances as antagonists of *Salmonella* in meat flour and bone flour, it was demonstrated that none of the products studied were effective in preventing the spread of the bacteria, with the exception of formalin at concentration over 0.1% (Fanelli et al., 1970).

Samuelson et al. (1985) measured the effect of lyzosime and etilen-diamine-tetraacetic acid on *Salmonella* growing over chicken meat, and concluded that both caused a significant reduction on the number of viable organisms.

The induced molting rejuvenates laying hens for a second or third cycle of production, resulting in higher egg production, heavier egg weight, and improvements in egg quality parameters, such as albumen height, shell thickness, and specific gravity (Len et al., 1964; Lee, 1982; Baker et al., 1983; Bell, 2003).

In several countries, the production of laying hens, is an industry that continues to implement induced molting to extend egg production in commercial laying flocks. Achieving an optimal molting requires dietary manipulation to cause a complete regression of the reproductive organs and cessation of egg production. This is followed by rejuvenation and initiation of an additional laying cycle. Currently, feed withdrawal is the primary means to initiate molt and is regarded as an optimal approach for achieving post-molt performance. However, removal of feed can lead to potential physiological stress in laying hens as well as an increased susceptibility to *S. enteritidis* colonization and invasion (Park et al., 2004).

The objective of the present study was the detection of *Salmonella spp.* in digestive tract, feces and cloacae of 3rd cycle laying hens in producing eggs by induced molt.

MATERIALS AND METHODS

A total of 30 randomly laying hens selected, at the Poultry Research Unit of the College of Veterinary Medicine, Universidad Autónoma de Nuevo León, were used for the present study. These birds were subjected to normal sanitary controls and had access to

Table 1. Numeration and identification of cages and hens of third cycle.

Number of cage	Number of identification of each bird*
1	9,3,2
2	38,35,36
3	29,8,37
4	11,12,30
5	20, 18, 14
6	24, 25, 26
7	15, 16, 1
8	17, 21, 4
9	10, 19, 23
10	22, 28, 27

*Randomlyselected.

Table 2. Growth in Bismuth-sulfite agar of samples taken from feces of laying hens on third production cycle.

Sample number	Site		Percentage	
	(+)	(-)	(+)	(-)
1	Zero	All	0	100
2	5, 8, 10	1, 2, 3, 4, 6, 7, 9	30	70
3	Zero	All	0	0
4	Zero	All	0	0
5	10	1, 2, 3, 4, 5, 6, 7, 8, 9	10	90

laying hen commercial feed with 14% crude protein. Bird control number and cage identification number are shown in Table 1.

The experiment consisted of the following phases: 1, Sampling of feces from cage floor and direct of cloaca. Ten places at the floor under the cages were marked and numbered, from which feces samples were taken once a week (Monday to Friday) for 5 weeks and plastic bags were used. Afterwards, a weekly laboratory analysis was performed to detect bacteria, and from this procedure, a total period of 5 weeks sampling and an total of 50 samples was used. For the cloaca sampling, five birds identified with numbers 9, 14, 20, 28 and 38 were utilized for 6 days (Monday to saturday) for a total of 30 samples using sterile hyssops and gloves. These samples were also analyzed by microbiological methods; 2, sampling of tissues at necropsy, was conducted in 30 randomly selected birds; samples were taken from the caecum, duodenal loop, liver, spleen, and gallbladder. Tissues recovered were subjected to microbiological analysis after mixing and homogenizing of the tissues collected under aseptic conditions.

Isolation of bacteria was achieved from inoculating 1 g of intestinal content in 3 ml of lactose culture medium, followed by incubation at 35-37°C for 24 h. Later, 1 ml was extracted, inoculated in 10 ml of tetrathionate medium and incubated for 24 to 48 h at 35-37°C. After this procedure, on Petri dishes bismuth sulfite agar were sown and incubated for 24 48 h at 35-37°C. Necropsy samples were cut into small fragments and cultured as mentioned above. Positive cultures were incubated for additional 24 h.

Bacterial species were identified by biochemical methods used for the Enterobacteriaceae family, such as TSI, LIA, SIM, urea, citrate, gelatin and MV-RP. Data collected in phase 1 were analyzed with the Chi-square test.

RESULTS

None of the 250 bacteriological cultures were positive to *Salmonella* spp., since all 9 strains isolated from *bismuth sulfite agar*, did not have characteristics comparable to *Salmonella*. Those bacteria showed positive results in Indole production with sulfhydric, indole, and mobility (SIM) test. Percentage positives to ASB was 3.5% from the total of analyzed samples.

Table 2 shows the results of bacterial culture growth-bismuth sulfite agar (ASB) of the feces samples taken from sites under the cages of laying birds in the third cycle. Only, in 2 sampling (in sites 5, 8, and 10) and 5 (in site 10), were the Bismuth sulfite agar culture positive, although they were not significant (P> 0.05), statistically.

Data taken from ASB cultures from cloaca samples of 5 birds chosen at random showed that only the sixth sampling was positive for bird number 28. This result was not significant (P > 0.05), since the percentage of positives was 0.04% (Table 3).

Table 4 shows the data on growth in *bismuth sulfite agar* of samples taken from organs during necropsy that were divided into two groups of birds. In the first group, the spleen samples were negative, whereas on group 2, they were positive; duodenal loop samples were positive for both groups as well as for caecum samples, which

Table 3. Growth in Bismuth-sulfite agar of samples from cloaca of laying hens on third production cycle.

Sample number	Total number of birds	Number of birds sampled randomly	Number of positive samples	Percent of positive
16	30	5 (9,14,20, 28, 38)	1 (No. 28) and at sixth sampling	0.04 %

Data analyzed by Xi squared test (Xi^2).

Table 4. Growth in Bismuth-sulfite-agar samples of organ taken from necropsies of laying hens in the third cycle.

Organ	Sampling number 1	Sampling number 2
Spleen	-	+
Duodenal loop	+	+
Caecum	+	+
Ovary	+	-
Liver	+	-
Gallbladder	-	-
Total	(Positives): 4	(Positives): 3

Table 5. Biochemical tests of positive samples in bismuth-sulfite agar performed in feces (S 2 y 5) and cloaca (S 6) of laying hens on third production cycle.

Test	Feces		Cloacae
	Sampling 2	Sampling 5	Sampling 6
Indol	+	+	+
Motility	+	+	+
H_2S*	+	+	-
LIA	+	+	-
TSI	R/R+	R/R+	A/A-
Urea	-	-	-
Simmons citrate	-	-	-
Nítrate	+	+	+
Gelatin (22ºC)	-	-	-
Metilo red	+	+	+
Voges- Proskauer	-	-	-

R, Alkaline: from orange-reddish to red; A, Acid, the culture remains yellow. *Measured both in SIM as in TSI.

were positive in both groups also.

Data on cultures ovary and liver were positive in the first sampling and negative for the second. Cultures of gallbladder were negative for both samplings. In general, percentage of positive organs for group 1 was 66.66% and for group 2 was 50%.

Table 5 shows the biochemical test of positive samples in ASB from feces and cloaca, where it is established that according to the Tables for Enterobacteria identification with biochemical reactions, organisms present were *E. coli* and *Edwardsiella spp* ,but not *Salmonella spp* .It can be noticed that the colony obtained from cloacae had no reaction with sulfuric acid and fermented sugars, changing the culture media to yellow (acidic).

Biochemical tests performed for samples positive to ASB (Table 6) from organ showed that *Edwasdsiella spp.* was present.

DISCUSSION

Importance of salmonellosis in both animal and human health has been highlightedfor a long time (Acha and Szyfres, 2001). Despite that all domestic animals, espe-

Table 6. Biochemical test of positive samples in Bismuth-sulfite agar culture, performed from organs of laying hens on third production cycle.

Test	Caecum	Ovary	Liver	Spleen	Duodenal loop	Gallbladder
Indol	+	+	+	+	+	+
Motility	+	+	+	+	+	+
H^2S*	+	+	+	+	+	+
LIA	-*	+	+	+	+	+
TSI (R/R)	+	+	+	+	+	+
Urea	-	-	-	-	-	-
Simmons citrate	-	-	-	-	-	-
Nitrate	+	+	+	+	+	+
Gelatin (22ºC)	-	-	-	-	-	-
Metilo red	+	+	+	+	+	+
Voges- Proskauer	-	-	-	-	-	-

R, Alkaline; *****This is considered *Edwardsiella spp.*, which ferments sugars slowly, and metabolizes it, and there are some strains that do not decarboxylate lysine (Cowan and Steel, 1975).

cially the young, are susceptible to this bacteria, it is considered that adult animals are the source of this disease in humans, specially the bird that can host *S. typhimurium* as a pathogen serotype (Acha and Szyfres, 2001).

The present work, attempted the isolation of *Salmonella* species from the third cycle laying hens, assuming that if they are "healthy" carriers, the increased life span of these animals as a commercial practice may enable a continued spread of *Salmonella spp.* to the habitat. However, because the isolation of the bacteria from this group of birds was not possible, it may mean that they do not come in contact with it through their lives or that conditions present at the gastrointestinal tract in birds of this age do not favors proliferation of *Salmonella* in amounts that allow its isolation with the procedures followed in this study.

Other studies report the presence of *S. enteritidis* during forced molt in some organs in hens, and the authors conclude that this was due to the decrease of feed and nutrient (McReynolds et al., 2006). Same conclusions were observed in the present work.

Moreover, Holt (2003) concluded that the molting increase indicates the presence of *Salmonella*, and in its research hreports indicate that the moulted hens excreted significantly higher *S*. *enteritidis* numbers in the faeces and possess greater levels of *S. enteritidis* in the internal organs (Holt, 2003). This is different to that reported in this study.

Growth of bacterial colonies in *bismuth sulfite agar* did not show characteristics compatible with *Salmonella spp.* (Merck, 1994). However, they were subjected to biochemical tests since the possibility existed for atypical *Salmonella* strains in ABS; the results for enterobacteria (Carter, 1985) indicated the presence of other microorganisms (*Edwardsiella* spp and *E. coli*). Isolation of *Edwardsiella spp.* from internal organs most likely was due to sample contamination during handling. Also, the mentioned finding must be taken with care since no other studies exist indicates the isolation of this bacteria.

The present work was realized with birds that were relatively isolated from other animal species (both from poultry farms and wild animals); therefore, this may be the reason for the absence of *Salmonella spp.* and it may not be discounted that third cycle laying hens, under different conditions could be carriers of the disease. This result indicates the role of a group of animals in maintaining *Salmonella spp.* in the environment.

Conclusions obtained from the present research are that it was not possible to isolate *Salmonella spp.* from feces and internal organs of the third cycle laying hens. The isolated bacteria were *Edwardsiella spp*. and *E. coli* based on biochemical specific tests.

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Full Length Research Paper

Incidence and anti-microbial resistance profile of Group B *Streptococcus* (GBS) infection in pregnant women in Nsukka, Enugu State, Nigeria

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This study was performed to determine the incidence and antimicrobial resistance profile of Group B *Streptococcus* in pregnant women in Nsukka, Enugu State. From January 2010 to July 2011, vaginal swab and rectal swab samples were obtained from 200 pregnant women of gestational age from 24 to 37 weeks that were attending antenatal clinic at Bishop Shanahan Memorial Hospital and Nsukka District Hospital. Vaginal and rectal cultures for the isolation and identification of Group B *Streptococcus* (GBS) were carried out according to standard microbiological methods. The Kirby-Bauer disk-diffusion method was employed to determine antibiograms of GBS isolates. Samples were also obtained from 200 non-pregnant women. The carriage rate of GBS among pregnant women was 18.00%, while in non-pregnant women it was 5.5%. Statistical analyses proved the difference to be significant (P<0.05). A total of 58 GBS isolates were used for *in vitro* susceptibility test to ampicillin and cloxacillin, amoxycillin, cefuroxine, ceftriaxone, erythromycin, ciprofloxacin, streptomycin, Pefloxacin, sulphamethoxazole and trimethoprim and gentamycin. No resistance to Ampicillin and Cloxacillin, amoxycillin, cefuroxine and ceftriaxone was found. Of the isolates examined, 6.70, 8.62, 8.62, 18.95, 32.75, 50.00% were resistance to ciprofloxacin, erythromycin, pefloxacin, streptomycin, sulphamethoxazole and trimethoprin and gentamycin, respectively.

Key words: Group B *Streptococcus* (GBS), incidence, anti-microbial resistance profiles, pregnant women, Nsukka.

INTRODUCTION

Streptococcus agalactiae is a normal flora of the female genital tract and an important cause of neonatal sepsis, meningitis and pneumonia (Tor-Udon et al., 2006; Altay et al., 2011). Group B Streptococci (GBS) are facultative, Gram positive cocci. Some strains of GBS are β-hemolytic and produce zones of hemolysis that are slightly larger than the colonies (1-2 mm in diameter).

GBS is present in up to one-third of women of child bearing age, and one in every thousand live births is affected by group B Streptococcal infection. Maternal GBS colonization is the most important risk factor for developing disease in the newborn (Baker and Edwards, 2001; Salah and Abouzeid, 2009). Maternal colonization can initiate the process of labour and cause preterm labour. Group B *Streptococcus* can ascend via lower genital organs to the uterus and may cause fetal membrane inflammation which leads to preterm rupture of membrane and labour (MacGregor and French, 2000; Quiroga et al., 2008). This vertical transmission of GBS from mother to infant is the most common mode of transmission.

In pregnant women that are highly colonized GBS can

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cause bladder infection, womb infection and still birth. The baby may develop symptoms of GBS disease in the first week of life. The symptoms include respiratory distress, sepsis among other (CDC, 2010). However, efforts are being made to reduce the incidence of early onset disease. It was reported that intrapartum chemophrophylaxis decreases the incidence of early onset disease from 1.7 to 0.6 per 1000 life birth (Schrag et al., 2002; CDC, 2010; Onipede et al., 2012). Adequate treatment and control required a good knowledge of the species involved and their susceptibility to antimicrobial agents.

The increased use of antimicrobials for prophylaxis has raised concerns regarding the emergence of resistance (Joyce et al., 2001). Penicillin has been the drug of choice for prophylaxis and treatment of GBS disease and as at 1995, resistance to this drug had not been reported (Andrew et al., 2000; CDC, 2010; Altay et al., 2011). However, macrolides are the recommended second line agents and the first alternative in mothers with penicillin allergy. In recent times, GBS strains that are resistant to macrolides have emerged and these strains have also shown resistance to other antibiotics such as erythromycin and clindamycin (Priscila et al., 2011). Resistance rates are reported to vary with geographical location (Joyce et al., 2001).

This study aimed at determining the incidence of GBS in pregnant women in Nsukka and studying the antimicrobial resistance patterns of the isolates.

MATERIALS AND METHODS

Study population and sampling

From January 2010 to July 2011, vaginal and rectal swabs samples were collected from 200 pregnant women of gestational age from 24 weeks to 37 weeks that were attending antenatal clinic at Bishop Shanahan Memorial Hospital and Nsukka District Hospital. Also, two hundred non-pregnant women were sampled from female hostels in University of Nigeria, Nsukka and some laboratories within Nsukka.

Sample collection

Vaginal swabs and rectal swabs were collected under aseptic condition from both pregnant and non-pregnant women following their informed consent and the ethical approval of the study. The vaginal and rectal swabs were collected using sterile cotton - tipped wooden swab sticks (Evepon industries Limited, NAFDAC REG. NO. 03-0482). These samples were put in a cold box with frozen ice packs and transported to the laboratory where they were cultured the same day.

Isolation and identification of organism

Vaginal and rectal swabs were inoculated on Todd-Hewitt broth (Oxoid, UK) which was supplemented with 8 µg/ml of gentamicin sulfate and 15 µg/ml of nalidixic acid and incubated for 18 - 24 hours at 37°C. The next day, 5 µl of the broth culture from each of the bijou bottles was inoculated onto a sterile selective GBS agar base medium (Oxoid U.K.) which was supplemented with 5% horse serum. A sterile glass rod was used to spread the inoculum over the entire surface of the GBS agar plate, to ensure an even distribution of the inoculum. The plates were then incubated at 35-37°C under anaerobic condition in an anaerobic jar with gas pack (Code No. AN0035A, from Oxoid) and read for the presence of orange-pigmented colonies after 18-24 h. Negative plates were re-inculcated for an additional 24-48 h before being discarded.

Isolates having orange pigment were subcultured onto sterile Blood agar plates and incubated for 18-24 h at 37°C in an atmosphere containing 5% carbondioxide. Following incubation, colonies were examined for the presence of Beta - hemolysis. Thereafter, β-hemolytic colonies from the blood agar were characterized using standard microbiological methods including colony morphology, Gram staining, catalase test, Christie, Atkins and Munch - Peterson (Camp test) and latex agglutination test using DRO587 latex grouping Reagent B (Oxoid Streptococcal Grouping Kit, RG 24 8pw U.K.), as described by (Cheesbrough, 2004).

Antimicrobial susceptibility test

A total of 58 GBS strains were used for *in vitro* susceptibility test. Susceptibility test to ten antibiotics namely- Ampicillin and Cloxacillin, Amoxycillin, Cefuroxine, Ceftriaxone, Erythromycin, Ciprofloxacin, Streptomycin, Pefloxacin, Sulphamethoxzole, Trimethoprim and Gentamycin were carried out on Mueller-Hinton agar supplemented with 5% sheep blood, using the disc diffusion technique. The isolates were considered susceptible or resistant according the zones of inhibition recommended by the clinical and laboratory standard institute (CLSI) (2007).

Data analysis

The data were analyzed using statistical package for the social sciences (SPSS). The relationship between values was tested using analysis of variance, chi-square and student t-test. The level of significance was taken at p-value < 0.05.

RESULTS

The incidence of GBS was 18.00% in pregnant women, and 5.50% in non-pregnant women. Statistical analysis proved the difference to be significant ($P < 0.05$). The percentage occurrence of GBS from vaginal swabs and rectal swabs samples of pregnant women were 8.50 and 8.50%, respectively, while in non-pregnant women percentage occurrence of GBS isolates were: vaginal, 2.50% and rectal 2.00% (Figure 1). GBS colonization rates in pregnant women of different age groups were: 15-20 years, 19.05%; 21-25 years, 18.75%; 26-30 years, 15.58% and 31-45 years, 21.05% (Figure 2). There was no significant difference ($P>0.05$) in these values. In non-pregnant women, Group B *Streptococcus* colonization was highest in women of age between 21-25 years, 9.86% (Figure 2). Based on parity, the GBS colonization rates were higher in pregnant women within their second pregnancy (25.53%) and third pregnancy (21.74%) than in those within their first pregnancy (15.31%) and fourth pregnancy (5.85%) (Figure 3). However, these differences were not statistically significant. The susceptibilities of the GBS isolates to all the antibiotics tested

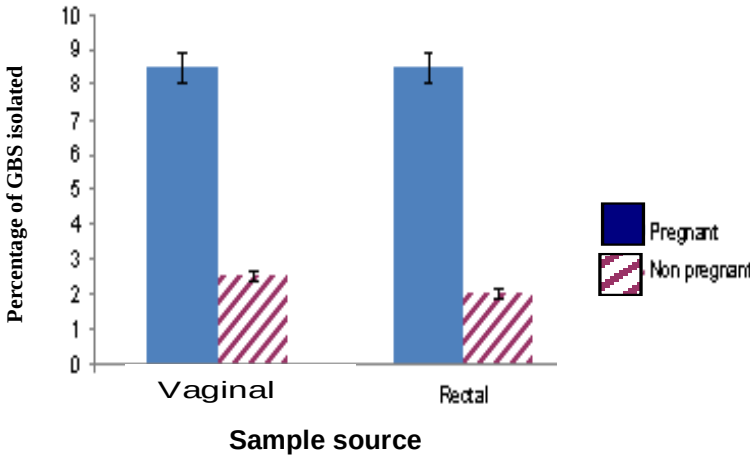

Figure 1. Percentage occurrence of Group B *Streptococcus* according to sample source (pregnant and non-pregnant women). *The error bars indicate standard deviation.

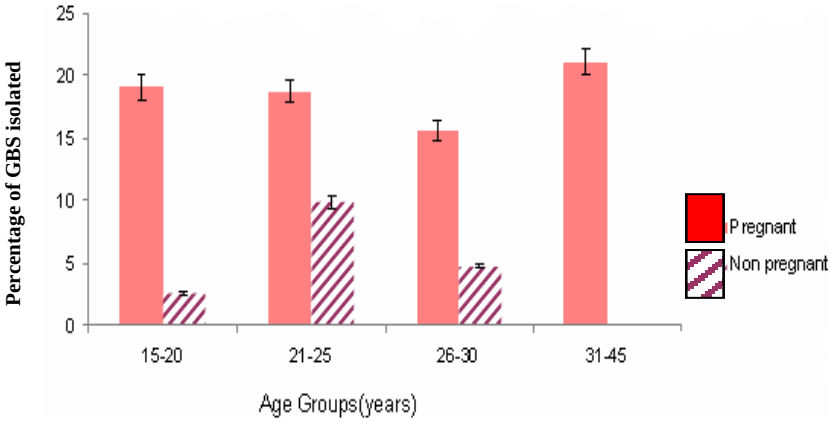

Figure 2. Percentage occurrence of Group B *Streptococcus* carriage rates in pregnant and non-pregnant women within different age brackets. *The error bars indicate standard deviation.

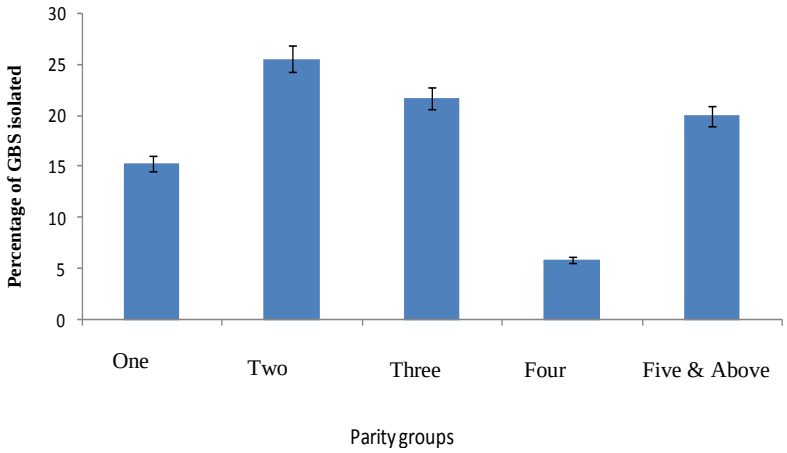

Figure 3. Group B *Streptococcus* (GBS) carriage rates in different parity groups. *The error bars indicate standard deviation.

Table 1. Antibiotic susceptibility profiles of group B *Streptococcus* isolates.

Antibiotic	Susceptible (%)	Intermediate (%)	Resistance (%)
Cefuroxine	58 (100)	-	-
Ceftriaxone	58 (100)	-	-
Amoxycillin	58 (100)	-	-
Ampicillin + cloxacillin	58 (100)	-	-
Erythromycin	53 (91.37)	-	5 (8.60)
Ciprofloxacin	54 (93.10)	-	4 (6.70)
Pefloxacin	53 (91.37)	1 (1.72)	4 (6. 70)
Streptomycin	47 (81.08)	9 (15.51)	2 (3. 40)
Sulphamethoxazole + trimethoprim	39 (69.24)	9 (15.51)	10 (17.24)
Gentamycin	29 (50%)	6 (10.34)	23(39.66)

are summarized in Table 1. All GBS were susceptible to ampicillin and cloxacillin, amoxycillin, cefuroxine and ceftriaxone. 91.37, 93.10 and 91.37%, of the isolates were considered susceptible to erythromycin, ciproflo-xacin and pefloxacin respectively. Resistance to erythromycin, ciprofloxacin, pefloxacin, streptomycin, sulphamethoxazole and trimethoprim and gentamycin was found to be 8.6, 6.7, 8.6, 18.95, 32.75 and 50.00%, respectively.

DISCUSSION

Group B *Streptococcus* (GBS) is an important cause of infection in pregnant women and their newborn in many countries, but there is a paucity of data available from Africa (Collins et al., 1998; Trujillo et al., 1990). Maternal Group B *Streptococcus* colonization continues to be the most important risk factor for developing disease in the newborn (Baker and Edward, 2001; Elbaradie et al., 2009). Most data on maternal Group B *Streptococcus* colonization over the years has come from Europe and North America and to date only Zimbabwe and Malawi in Africa have an active research programme on GBS colonization and the burden of the disease (Dzowela et al., 2005). The incidence of GBS among pregnant women in this study was 18.00% which is in agreement with 16.50% reported in Malawi (Dzowela et al., 2005), 19.00% in Ivory Coast, 22.00% in Gambia (Stoll and Schuchat, 1998), 20-32% in Zimbabwe (Moyo, 2002) and 11.3% in Ile-Ife, Nigeria (Onipede et al., 2012). However, some other investigators have reported lower values e.g. 9.50% in Korsa (Uh et al., 2001). In non pregnant women the incidence was 5.50%. This is also in agreement with reports of other authors that have shown higher incidence in pregnant than non-pregnant women (Farley, 2001; Blancas et al., 2004).

It was observed that the majority of pregnant women enrolled in this study were between the ages of 21 to 30 years. The incidence of Group B *Streptococcus* in pregnant women within these various age range was highest in women of age between 31 - 45 years followed by those between 15 - 20 years. The pattern of GBS isolated from different age range in this study showed that the incidence was not age dependent. This agrees with some authors who reported that the distribution of isolates from asymptomatically colonized pregnant women was irrespective of age (Baker et al., 1997; Timothy et al., 1998). In non-pregnant women, Group B *Streptococcus* colonization was highest in women of age between 21-25 years. This may be attributed to the fact that young women in this age bracket are more sexually active than older women. It has been reported that GBS is more common in sexually active women including both frequent intercourse and multiple sex partners (Meyn et al., 2002).

GBS colonization was evaluated in relation to parity and the highest incidence was observed among group two (25.53%) and group three (21.74%) and the lowest incidence in group four (5.88%). This variation within the various parity groups can be explained on the basis of the difference in hygienic status of different populations.

In this study, we found no resistance to Ampicillin and Cloxacillin, Amoxycillin, Cefuroxine and Ceftriaxone. So, Penicillin or Ampicillin remains the drugs of choice for intrapartum antibiotic prophylaxis for GBS colonization in pregnant women.

Erythromycin is the drug of choice for women with serious penicillin allergy who are colonized with GBS (CDC, 2010). An increase in resistance of GBS to erythromycin has been reported (Dipersio, 2006; Samar et al., 2012). We found that 8.6% of the isolates were resistant to erythromycin. This rate of resistance to erythromycin observed among the GBS isolates in this study is consistent with reports from other authors (Di Bartolomeo et al., 2005; Mollerach et al., 2007; Aziz et al., 2011). The rate of erythromycin resistance in the GBS isolates strongly supports the current CDC recommend-ddation that antibiotic susceptibility test should be performed if erythromycin therapy is needed to prevent neonatal GBS infection (Heclan et al., 2004).

Resistance to quinolones (pefloxacin and ciprofloxacin) has only recently been described for GBS (Quroga et al., 2008; Tazi et al., 2008). In our study, we identified 6.70 and 8.62% of the isolates resistant to ciprofloxacin and pefloxacin, respectively.

With more widespread use of antibiotics, selection of antibiotic resistant GBS may occur. If resistance continues to be identified and increase, surveillance of antibiotic resistance patterns among several antimicrobial classes will be important in determining optimal prophylaxis and treatment of GBS infections.

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Full Length Research Paper

Evaluation of *Lactobacillus* and *Bacillus*-based probiotics as alternatives to antibiotics in enteric microbial challenged weaned piglets

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The present study investigates the efficacies of two probiotic products as alternative to antibiotics on growth performance, nutrient digestibility, immunity, and fecal microbiota in piglets challenged with *Salmonella enterica* serovar Typhimurium KCTC 2515 and *Escherichia coli* KCTC 2571. Ninety-six 28-day-old piglets were randomly allotted to four dietary treatments consisting of four replicate pens with six piglets each. The dietary treatments were: negative control (NC), positive control (PC, 0.002% apramycin), 0.5% *Lactobacillus* probiotic (P1), and 0.04% *Bacillus* probiotic (P2). Average daily gain (ADG) and average daily feed intake (ADFI) were improved by treatment with PC and P1, whereas, feed conversion ratio (FCR) was improved by treatment with P2 compared to NC ($P < 0.05$). Digestibility of dry matter, crude protein, and crude fat increased upon treatment with PC, P1, and P2 compared to NC. All dietary treatments showed significant reduction of fecal *Salmonella* and *E. coli* counts with an increase of *Lactobacillus* and *Bacillus* spp counts compared to NC ($P < 0.05$). The serum IgG level was elevated by P2 treatment compared to others ($P < 0.05$). Overall, both *Lactobacillus* and *Bacillus* probiotics had beneficial effects on weaned piglets under challenged condition and therefore, can be used as potential alternatives to antibiotics.

Key words: *Lactobacillus*, *Bacillus*, fecal microbiota, immunoglobulin, challenged piglet.

INTRODUCTION

One of the most important aspects of veterinary research is to improve the quality and delivery of safe livestock products (meat, milk and egg) for human consumption. Swine meat, widely consumed worldwide, can be a source of food borne pathogens such as *Salmonella* and *Escherichia coli* (Korsak et al., 2003). Therefore, it becomes critical to the producers to identify the best methods to mitigate *Salmonella* and *E. coli* infection from pig meats. In the past, antibiotics were included at sub-therapeutic levels, acting as growth promoters (Antibiotics Growth Promoters, AGPs) and reducing the pathogen load (Dibner and Richards, 2005). However, there is a recent consumer rising trend in having AGPs removed from animal agriculture due to health and environmental issues, together with the increase of bacterial strain resistant against many human antibiotics. These concerns have resulted in the severe restriction or total elimination of antibiotics as growth promoters (EC, 2003) in many countries. Langlois et al. (1988) demonstrated that complete removal of antibiotics from animal production diminished resistance of lactose-fermenting fecal coliform bacteria. However, that has put tremendous pressure on the livestock industry to identify viable therapeutic alternatives against food borne pathogens, such as probiotics, which have used successfully in livestock feeds (Alexopoulos et al., 2004; Chen et al., 2005, 2006).

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Table 1. Ingredients and nutrient levels of basal diet.

Item	Value
Ingredients (%,as-fed basis)	
Yellow corn	45.15
Wheat	23.00
Wheat bran	4.00
Soybean meal	18.00
Limestone	0.98
Calcium phosphate	1.10
Salt	0.25
Vitamin premix[A]	0.55
Animal fat	2.50
Molasses	4.30
L-Lysine	0.17
Chemical composition (as fed basis)[B]	
ME (kcal/kg)	3265
Crude protein (%)	18.0
Ca (%)	0.70
Available phosphorus (%)	0.55
Lysine (%)	0.95
Methionine (%)	0.30

[A]Contains the following nutrients per kg of diet: vitamin (V) A 6000 IU; VD3 800 IU; VE 20 IU; VK3 2 mg; VB1 2 mg; VB2 4 mg; VB6 2 mg; VB12 1 mg; pantothenic acid 11 mg; niacin 10 mg; biotin 0.02 mg; Cu 21 mg; Fe 100 mg; Zn 60 mg; Mn 90 mg; I 1.0 mg; Co 0.3 mg; Se 0.3 mg. [B]Calculated values.

Probiotics refer to a group of non-pathogenic organisms that, when ingested in sufficient number, have beneficial effects on the health of the host (Reid et al., 2003). There are three main categories of organisms that are commonly referred to as probiotics: Lactic acid bacteria (LAB), spore-forming *Bacillus* spp., and yeast. *Lactobacilli* are non-pathogenic, Gram-positive bacteria as well as natural inhabitants of the porcine gastro-intestinal tract. Previous studies have discovered that native gut microbes can successfully prevent infection by *Salmonella* spp. (Nurmi and Rantala, 1973) and reduce shedding of pathogenic *E. coli* (Watkins et al., 1982).

However, their concentration decreases dramatically immediately after weaning (Huis in't Veld and Havenaar, 1993) which allows the proliferation of pathogenic bacteria. On the other hand, some *Bacillus* spp., with soil as their natural habitat, are used as probiotics, either alone or in combination (Hong et al., 2005). They cannot colonize in the gastrointestinal tract; but stimulate the growth of *Lactobacilli* through production of catalase and subtilisin (Hosoi et al., 2000). Several studies reported improvements in growth performance (Huang et al., 2004; Alexopoulos et al., 2004), nutrient digestibility (Shon et al., 2005; Chen et al., 2006), humoral and cell-mediated immune responses (Fernandes and Shahani, 1990; European food safety authority, 2010), and the microbial ecosystem (Huang et al., 2004; Baker et al., 2013) upon dietary supplementation with *Lactobacillus* or *Bacillus*-based probiotics, although inconsistencies in result have also been reported (Cromwell, 2001). The discrepancies observed can be attributed to different strains, dose levels, diet compositions, feeding strategies, age of animals, etc (Chesson, 1994). This indicates the need of specific studies to elucidate the efficacies of the several probiotic preparations.

This study was done to investigate the efficacy of *Lactobacillus*- and *Bacillus*-based probiotic preparations as an alternative to typical AGPs and assess their effects on growth performance, nutrient digestibility, immunity, and microbial ecology of weaned piglets challenged with *Salmonella enterica* serovar Typhimurium KCTC 2515 and *Escherichia coli* KCTC 2571.

MATERIALS AND METHODS

Experimental studies with piglets were approved by the Animal Care and Use Committee of Sunchon National University, Suncheon, Republic of Korea.

Source of probiotics

The *Lactobacillus*-based probiotic preparation, Avilac, used in the current experiment was manufactured by Daesung Microbiologica Labs Co., Ltd. (Seoul, Korea) and containing at least 10^{10} cfu of *Lactobacillus reuteri avibro*/kg of diet. The *Bacillus*-based probiotic, Bioplus 2B, was manufactured by Easy Bio System Inc. (Seoul, Korea) and containing *Bacillus subtilis* and *Bacillus licheniformis* both at 3.2×10^9 cfu/kg of diet.

Probiotic supplementation diet

A total of 96 three-line crossbred [(Landrace × Yorkshire) × Duroc] weaned piglets (28-days-old, average body weight of 8 kg) were housed for a period of 28 days. A completely randomized design was used with four treatments and four replicates (pens of 6 piglets with an equal sex ratio of three male and three female) per treatments, where piglets were allotted by body weight. Four dietary groups were formed; each consisted of 24 piglets (four replicates of six pigs per pen). The dietary groups included: basal diet without any supplement (NC; negative control), basal diet added with 0.002% apramycin (PC; positive control), basal diet added with 0.5% *Lactobacillus*-based probiotic (P1), and basal diet added with 0.04% *Bacilli*-based probiotic (P2). The dose levels of antibiotic and probiotics used in this experiment were determined in accordance with previous research (Ahmed et al., 2013; Harper et al., 1983; Gracia et al., 2004).

The antibiotic and probiotic products were mixed on a weight:weight ratio basis by replacing an equal amount of basal diet. The basal diet used in this experiment was in pellet form and was formulated to provide the nutrient requirements recommended by the NRC (1998). The ingredients and composition of the experimental diet are presented in Table 1. All pigs were housed in an environmentally controlled isolation trailer with a slatted plastic floor in 12 adjacent pens. Each pen was equipped with a one-sided

self-feeder and a nipple drinker to allow *ad libitum* access to feed and water throughout the experimental period. The target room temperature and humidity were 25°C and 60%, respectively. Individual pig body weights were measured at the beginning (day 1), middle (day 14), and end (day 28) of the experiment, and ADG was calculated. Feed consumption was recorded on a pen basis every other week, and the ADFI and FCR were calculated.

Oral challenge

The *S. enterica* serovar Typhimurium KCTC 2515 and *E. coli* KCTC 2571 used in the study are parts of the Korean Collection for Type Cultures (KCTC) and the stocks were purchased from the Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea. The bacterial cultures were prepared by growth in LB (Luria-Bertani) Broth (Difco, Detroit, MI, USA) for 24 h at 37°C by using 1% inoculum volume from the stocks. Tenfold dilution of the bacterial cultures were made prior to challenge and plated in agar media to enumerate the cell concentration per ml. All piglets were orally challenged with 5 ml of culture fluid containing 5.9×10^8 cfu/ml of *S. enterica* serovar Typhimurium KCTC 2515 and 2.3×10^8 cfu/ml of *E. coli* KCTC 2571 at the back of the oral cavity using a micropipette tip. Bacterial solution was slowly dribbled into piglet's throat in order to trigger the swallowing reflex and minimize the passage of inoculants into the lungs. The goal was to use the challenge as a model of post-weaning lag phase, which is manifested by an imbalanced microbiota in the intestine, resulting in poor growth performance and immunity. The piglets were housed in an environmentally controlled isolation trailer to prevent possible cross-contamination.

Sampling and measurement

A digestibility trial was conducted using chromium oxide (0.20%) as an indigestible marker (Fenton and Fenton, 1979). All piglets were fed diets mixed with chromium oxide (Cr_2O_3) on day 21, and fecal grab samples were collected from all pigs on day 28 and stored immediately in sealed plastic bags at -20°C until analysis. For chemical analysis, the fecal grab samples were dried in a force-air drying oven at 70°C for 72 h and then finely ground to pass through a 1 mm screen. Analyses of feed and fecal samples were done in accordance with the methods established by the AOAC (2000). The chromium concentration was measured with an atomic absorption spectrophotometer (Model AA-6200; Shimadzu Corp., Kyoto, Japan) in a cuvette blanked with distilled water at 440 nm. Standard curves were prepared by using a stock solution of pure Cr_2O_3 (100 mg/100 ml), diluted to several working standards of 5, 10, or 20 mg/100 ml and carrying them through each method. The optical density was plotted against milligrams of Cr_2O_3. The digestibility was then calculated using the following formula:

$$\text{Digestibility (\%)} = [1-\{(N_f \times C_d) / (N_d \times C_f)\}] \times 100$$, in which

N_f = Nutrient concentration in feces (%DM)
N_d = Nutrient concentration in diet (%DM)
C_f = Chromium concentration in feces (%DM)
C_d = Chromium concentration in diet (%DM)

For microbial analysis, two piglets (one male and one female) were identified from each pen by a double ear-tag on day 1 of the trial. Fresh fecal samples were collected on day 7, 14, 21 and 28 of the experiment, directly from the rectum of these piglets in sterile polyethylene bags, via manual stimulation of the internal and external anal sphincters, in order to avoid any additional contamination of the samples. The samples were than serially diluted 10-fold in sterile saline (0.9%). Microbial assay of fecal samples was carried out by culture techniques. The microbial groups analyzed were *S. typhimurium* [Salmonella-Shigella (SS) agar], *E. coli* [MacConkey (MAC) agar], *Lactobacillus* spp. [de Man, Rogosa and Sharpe (MRS) agar], and *Bacillus* spp. [Mannitol Egg Yolk Polymyxin (MYP) agar]. The microbial plates were inoculated with three dilutions each in duplicate. The agar plates were then incubated anaerobically at 37°C for 24 h, after which microbial colonies were immediately counted. Microflora enumerations were expressed as $\log^{10}$ cfu/mL.

For immunoglobulins quantification, blood samples were collected directly from the jugular vein on day 28 using a 22-gauge sterile needle in a 10 ml syringe and then transferred to a BD Vacutainer (Becton Dickinson, Franklin Lakes, NJ) without anticoagulant. The blood was then quickly transferred to a centrifuge tube and centrifuged for 15 min at 3,000 rpm (1610 × g) in a cold chamber (4°C). The sera were carefully removed to plastic vials and stored at -20°C until immunoglobulin analysis was performed. The concentrations of serum IgG, IgM, and IgA were assayed using Pig IgG (Cat. No. E100-104), IgM (Cat. No. E100-100), and IgA (Cat. No. E100-102) ELISA Quantitation Kits (BETHYL Laboratories Inc., USA), respectively, whereas TNF-α was assayed using a Porcine TNF-α Quantikine ELISA Kit (Cat. No. PTA00) according to manufacturer's instructions. Each experiment was run in duplicate, and the results represent the means of three experiments. The absorbance of each well was measured using a microplate reader (Thermo Lab Systems, Finland) at 450 nm (Correction wavelength, 570 nm). The results were expressed as mg/ml of serum.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) appropriate for a completely randomized design by using the general linear model procedures (GLM) of the SAS Institute Inc. (SAS, 2003). The pen was used as the experimental unit to analyze growth performance and nutrient digestibility, whereas an individual piglet was used as the experimental unit for analysis of serum immunoglobulins and fecal microbiota. Statistically significant effects were further analyzed, and means were compared using Duncan's multiple range tests. Probability values of $P < 0.05$ were considered as statistically significant, whereas $P < 0.10$ was considered a tendency.

RESULTS

Growth performances

The effects of probiotics on growth performance of weaned piglets are shown in Table 2. During phase 1 (days 0 to 14), ADG of piglets treated with P1 was greater ($P = 0.0004$) compared to NC, PC, or P2. On the other hand, during phase 2 (days 14 to 28) and the overall experimental period (days 0 to 28), ADG of piglets treated with PC and P1 were greater ($P < 0.0001$) compared to P2 or NC treatment. Moreover, ADG induced by P2 treatment was non-significantly higher compared to NC treatment during all phases.

During phase 1, phase 2, and the overall experimental period, ADFIs of the P1 and PC-supplemented groups were higher ($P < 0.0001$) compared to NC or P2 treatment, with P2 showing a non-significantly lower effect than NC. During phase 2 and the overall experimental

Table 2. Effects of *Lactobacillus*- and *Bacillus*-based probiotics on growth performance of challenged piglets.

Parameter[A]	Treatment[B]				SEM[C]	P-value
	NC	PC	P1	P2		
Initial BW (kg/piglet)	8.01	8.44	7.97	8.17	0.33	0.75
ADG (g/piglet)						
Phase 1 (day 0 to 14)	296^{c}	383^{b}	481^{a}	309^{c}	22.88	0.0004
Phase 2 (day 14 to 28)	191^{c}	362^{a}	298^{ab}	255^{bc}	21.51	0.001
Overall (day 0 to 28)	243^{b}	373^{a}	390^{a}	282^{b}	13.36	<.0001
ADFI (g/piglet)						
Phase 1(day 0 to 14)	451^{c}	492^{b}	670^{a}	440^{c}	8.90	<.0001
Phase 2 (day 14 to 28)	517^{c}	848^{a}	719^{b}	511^{c}	12.87	<.0001
Overall (day 0 to 28)	484^{b}	670^{a}	695^{a}	475^{b}	8.01	<.0001
FCR (feed/gain)						
Phase 1(day 0 to 14)	1.59	1.36	1.41	1.43	0.13	0.70
Phase 2 (day 14 to 28)	2.78^{a}	2.38^{ab}	2.42^{ab}	2.07^{b}	0.17	0.12
Overall (day 0 to 28)	2.00^{a}	1.81^{ab}	1.79^{ab}	1.69^{b}	0.08	0.08

^{a,b,c} Means in a row with no common superscripts differ significantly ($p < 0.05$) or tend to differ ($p < 0.10$). [A]BW, Body weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio. [B]NC: negative control; basal diet, PC: positive control; basal diet + 0.002% Apramycin, P1: basal diet + 0.5% *Lactobacillus*-based probiotics, P2: basal diet + 0.04% *Bacillus*-based probiotics. [C]Standard error of the means.

Table 3. Effects of *Lactobacillus*- and *Bacillus*-based probiotics on nutrient digestibility of challenged piglets.

Nutrient digestibility (%)	Treatment[A]				SEM[B]	P-values
	NC	PC	P1	P2		
Dry Matter	72.6^{c}	85.8^{a}	77.1^{b}	76.2^{b}	0.88	<.0001
Crude Protein	65.2^{c}	77.1^{a}	71.7^{b}	69.9^{b}	1.53	0.001
Crude Fat	66.3^{b}	78.4^{a}	76.9^{a}	78.6^{a}	1.18	<0.001
Crude Fiber	68.1^{b}	82.3^{a}	73.1^{b}	73.0^{b}	1.80	0.009
Crude Ash	45.3^{b}	66.7^{a}	53.4^{b}	47.7^{b}	2.47	0.002

^{a,b,c} Means in a row with no common superscripts differ significantly ($P < 0.05$). [A]NC: Negative control; basal diet, PC: Positive control; basal diet + 0.002% Apramycin, P1: basal diet + 0.5% *Lactobacillus*-based probiotics, P2: basal diet + 0.04% *Bacillus*-based probiotics. [B]Standard error of the means.

period, FCR of piglets treated with P2 was lower ($P < 0.10$) compared with other treatments, with PC and P1 showing intermediate effects.

Apparent nutrient digestibility

Apparent digestibility of dry matter (DM) and crude protein (CP) were greater ($P < 0.01$) in piglets treated with PC, P1, and P2 compared to NC, with P1 and P2 showing intermediate effects (Table 3). Digestibility of crude fat (EE) was elevated ($P < 0.01$) upon treatment with PC, P1, and P2 in relation to NC. Apparent digestibility of crude fiber (CF) and crude ash (CA) increased upon PC treatment ($P < 0.01$) compared to treatment with P1, P2, or NC.

Fecal microflora population

The results of the study on fecal microflora concentration are shown in Table 4. On day 7, piglets treated with P1

Table 4. Effects of *Lactobacillus*- and *Bacillus*-based probiotics on fecal microbial concentrations in challenged piglets (log_{10} cfu/ml).

Day post infection	Treatment[A]				SEM[B]	P-value
	NC	PC	P1	P2		
S. typhimurium						
D 7	4.09[a]	3.54[ab]	2.96[b]	4.04[a]	0.25	0.07
D 14	4.00[a]	1.92[b]	1.49[b]	3.34[a]	0.29	0.007
D 21	4.26[a]	0.77[c]	2.73[b]	3.03[b]	0.21	<.0001
D 28	4.17[a]	0.33[c]	1.85[b]	0.33[c]	0.25	<.0001
E. coli						
D 7	4.73	4.68	4.88	4.95	0.14	0.54
D 14	5.42	5.44	5.40	5.14	0.22	0.75
D 21	5.62[a]	4.60[b]	5.71[a]	5.07[b]	0.15	0.004
D 28	6.19[a]	4.97[b]	5.38[b]	5.48[b]	0.17	0.008
Lactobacillus spp.						
D 7	7.10[a]	6.38[b]	7.42[a]	7.28[a]	0.11	0.0005
D 14	7.24[ab]	7.03[ab]	7.40[a]	6.73[b]	0.15	0.91
D 21	7.19	6.91	7.20	7.21	0.18	0.70
D 28	6.71[b]	7.08[a]	7.26[a]	7.08[a]	0.10	0.03
Bacillus spp.						
D 7	5.91	6.01	6.16	6.05	0.20	0.87
D 14	6.33	6.53	6.06	6.48	0.23	0.52
D 21	6.51	6.40	6.49	6.36	0.14	0.86
D 28	6.35[b]	7.13[a]	6.93[a]	7.34[a]	0.11	0.002

[a,b,c] Means in a row with no common superscripts differ significantly ($P < 0.05$). [A]NC: Negative control; basal diet, PC: Positive control; basal diet + 0.002% Apramycin, P1: basal diet + 0.5% *Lactobacillus*-based probiotics, P2: basal diet + 0.04% [B]Standard error of the means.

showed lower ($P < 0.10$) fecal counts of *Salmonella* in relation to P2, PC, or NC. On day 14, fecal *Salmonella* counts were significantly reduced ($P < 0.01$) by PC and P1 treatments in relation to P2 or NC. On days 21 and 28, piglets treated with PC, P1, and P2 all showed lower ($P < 0.0001$) fecal *Salmonella* counts compared to NC treatment. Dietary supplementation of antibiotic (PC) or probiotic products (P1 and P2) had no effects on fecal counts of *E. coli* on days 7 and 14. On day 21, treatment with PC and P2 showed reduced ($P < 0.01$) *E. coli* counts compared to P1 or NC treatment. However, on day 28, piglets treated with PC, P1, and P2 all showed lower ($P < 0.01$) fecal *E. coli* counts in relation to NC treatment.

On days 7 and 14, PC treatment had reduced ($P < 0.01$) fecal counts of *Lactobacillus* spp. in relation to P1, P2, or NC ($P < 0.10$). Whereas the dietary treatments showed no effects on fecal *Lactobacillus* spp. counts on day 21. Fecal *Lactobacillus* spp. counts increased at day 28 ($P < 0.05$) following treatment with P1, P2, and PC compared to NC. There were no effects of dietary supplementation of antibiotics (PC) and probiotic products (P1 and P2) on fecal *Bacillus* spp. counts in piglets on days 7, 14 and 21. However, on day 28, piglets treated with PC, P1, and P2 all showed higher ($P < 0.01$) fecal *Bacillus* spp. counts in relation to NC.

Serum levels of IgG, IgM, IgA and TNF-α

Table 5 shows the effects of dietary treatments on serum immunoglobulin and TNF-α concentrations in challenged piglets. The serum IgG concentration in piglets treated with P2 was greater ($P < 0.01$) compared to those treated with NC, PC, or P1. However, the concentrations of IgM and IgA were unaffected by the dietary treatments ($P > 0.05$). Antibiotics and both probiotic groups showed significantly lower concentrations of serum TNF-α in relation to control ($P < 0.0001$).

DISCUSSION

A well balanced gut microbiota is able to positively affect

Table 5. Effects of *Lactobacillus*- and *Bacillus*-based probiotics on serum immunoglobulins and TNF-α concentration of challenged piglets.

Parameter	Treatment[A]				SEM[B]	P-values
	NC	PC	P1	P2		
IgG (mg/mL)	409[b]	366[b]	417[b]	527[a]	17.5	0.001
IgM (mg/mL)	30.2	29.9	30.8	30.2	0.47	0.66
IgA (mg/mL)	6.33	5.80	6.10	6.50	0.42	0.69
TNF-α (pg/mL)	133[a]	98.7[b]	101[b]	99.7[b]	1.14	<.0001

[a,b]Means in a row with no common superscripts differ significantly (P < 0.05). [A]NC: Negative control; basal diet, PC: Positive control; basal diet + 0.002% Apramycin, P1: basal diet + 0.5% *Lactobacillus*-based probiotics, P2: basal diet + 0.04% *Bacillus*-based probiotics. [B]Standard error of the means.

the integrity of the intestinal barrier against pathogen colonization through its protective and metabolic function and can stimulate the immune system in an anti-inflammmatory manner (Gaggia et al., 2010). However, physiological or psychological stresses such as weaning lead to dysfunction of the intestinal barrier function by negatively altering gut microbial composition (Gareau et al., 2009). Probiotics are mainly used to reinforce or re-establish the gut microbial balance, especially when hosts are confronted with challenges or stress (Vanbelle, 2001), generally associated with poor growth rate and immunity. Probiotic bacteria such as *Lactobacilli* and *Bacilli* have been shown to improve growth performance of pigs by maintaining the intestinal microbial balance (Alexopoulos et al., 2004; Shon et al., 2005). In the current study, we examined the efficacy of *Lactobacillus*- or *Bacillus*-based probiotics for improving the growth performance, nutrient digestibility, microbial ecosystem, and immune response of weaned piglets challenged with the enteric pathogens *Salmonella enterica* serovar Typhimurium and *E. coli*. The antibiotic apramycin was used as a positive control with the objective of evaluating the efficacies of the probiotic products as alternatives to AGPS.

In the present study, application of *Lactobacillus*-based probiotics and antibiotics resulted in improvement of ADG and ADFI, whereas *Bacillus*-based probiotics resulted in improvement of FCR. These beneficial effects of antibiotic and probiotic supplementation on growth performance are consistent with the results of Cromwell (2001), Shon et al. (2005), and Wang et al. (2011). *Lactobacilli* bacteria are natural inhabitants of the gastrointestinal tracts of piglets. Their metabolites, including lactic acid, digestive enzymes etc., stimulate gastrointestinal peristalsis and promote apparent nutrient digestibility, which improves the appetites of piglets and maintains microbial equilibrium in the intestine (Wang et al., 2011). However, it was previously shown that *Lactobacilli* counts decline dramatically immediately after weaning (Huis in't Veld and Havenaar, 1993), resulting in microflora imbalance, digestive disturbance and poor performance of piglets. Therefore dietary supplementation of *Lactobacillus*-based probiotic could be beneficial (Djouzi et al., 1997). In the present study, increased ADG and ADFI in piglets fed *Lactobacillus*-based probiotics could be attributed to improved digestibility of nutrients and microbial ecology in the intestine. Supplementation of *Bacillus*-based probiotic had no significant effect on ADG and ADFI of weaned piglets which is partially consistent with previous studies (Kritas and Morrison, 2004; Min et al., 2004). However, these results have not always been consistent. For example, Gracia et al. (2004) reported improved ADG and ADFI during prestarter and overall prestarter-finishing period by dietary 0.04% Bioplus 2B. However, Robert and Gabriel (2006) reported that addition of 0.04% Bioplus 2B at different periods had different effects on the ADG of young pigs. In our study, lower improvement in ADG in *Bacillus*-probiotic treatments may be due to the short treatment period (Wang et al., 2009). The numerical reduction of ADFI in the *Bacillus*-based probiotic-treated group can be considered as a contributing factor in the increased feed to gain ratio. The reduced feed intake and improved FCR with no effect on weight gain indicates that the *Bacillus* probiotics indeed exerted some beneficial effects in the piglets. *Bacillus* can produce some useful enzymes such as amylase, protease (Ohno et al., 1995), that improves the apparent digestibility of complex carbohydrates and proteins, thus increasing the FCR (Anjum et al., 2005).

Supplementation of probiotic products and apramycin improved the apparent digestibility of DM, CP and EE. In agreement with the findings of the present study, Meng et al. (2010), and Shim et al. (2010) reported greater apparent digestibility of DM and CP in pigs and broilers supplemented with probiotics complex. Min et al. (2004) reported the positive effects of 0.04% Bioplus 2B in the DM and N digestibilities of nursery pigs. Conversely, Shon et al. (2005) and Wang et al. (2009) reported no improvement in nutrient digestibility of growing pigs by dietary *Lactobacillus* or *Bacillus*-based probiotic supplementation, respectively. Improvement of apparent digestibility of nutrients by probiotics and antibioitcs could be attributed to increased nutrient availability for absorption via suppression of growth and metabolic activities of

harmful gut microbiota along with simultaneous alteration of the intestinal morphology (Shim et al., 2010). *Bacillus* and *Lactobacillus* are also known to increase the rate of glucose transport, intestinal villous height, and crypt depth ratio (Breves et al., 2000; Rao and Wang, 2010), which may have contributed to improved nutrient uptake in pigs. Moreover, probiotic products may compete with other intestinal microorganisms for nutrients or result in production of antibacterial substances (Hentges, 1992) if continuously administered to the animals, which would explain the results regarding nutrient digestibility.

Application of either antibiotic or probiotic treatments resulted in reduced numbers of fecal *Salmonella* and *E. coli* as well as increased *Lactobacillus* spp. and *Bacillus* spp. counts compared to control. It has been reported that probiotic bacteria maintain normal gut microflora in two ways: competitive exclusion or antagonism. Once established in the gut, probiotic bacteria may produce compounds with bactericidal or bacteristatic properties (bacteriocins) such as organic acids, hydrogen peroxide, lactoferrin, etc (Jin et al., 1997). These substances are thought to inhibit the growth of pathogenic bacteria by reducing the pH in the gut (FEFANA EU Feed Additives and Premixtures Association, 2005). Researchers found that *L. reuteri* can secrete sufficient amounts of a broad spectrum antibiotic substance, reuterin, resulting in the desired anti-microbial effects (Talarico et al., 1988). *Lactobacilli* themselves can also colonize the gut mucosa to form a biological barrier to pathogenic microbes. Huang et al. (2004) reported that *Lactobacilli* isolated from weaned pigs are able to reduce gut *E. coli* and increase gut *Lactobacilli* counts. Reduction of fecal shedding of *Salmonella* was also observed upon dietary supplementation of a mixture of probiotics (mainly *Lactobacilli*) in weaned pigs (Casey et al., 2007).

Although *Bacillus* spp. is not a principal member of the normal intestinal flora and could not colonize the intestine for long periods, it consumes oxygen rapidly and reduces pH, which favors *Lactobacilli* and inhibits *E. coli* and *Salmonella* (Wu et al., 2011). On the other hand, the digestive enzymes secreted by *Bacillus* spp. have limited effects on improving production performance in animals, although the various nutrients yielded by these enzymes may contribute to population changes in the fecal microflora to some extent. The increased number of *Lactobacillus* and *Bacillus* counts in the antibiotic-treated group during the last week of the experiment can be attributed to the development of resistance against antibiotics (Sarra et al., 1982).

The capacity of probiotics to modulate the immune system is one of the more recent developments in the livestock field. In the present study, immune responses were evaluated by determining levels of serum immunoglobulins (IgG, IgM, and IgA) and the cytokine TNF-α. Our results showed that serum IgG values significantly increased in the *Bacillus*-based probiotics-treated group. Our results are consistent with a report by Pătrăscanu et al. (2011), who observed increased IgG and IgM levels in pregnant sows supplemented with *Bacillus*-based probiotics Bioplus 2B®.

Chen et al. (2005) also observed increased IgG levels in growing pigs upon supplementation of a probiotics complex (*Lactobacillus acidophilus*, 1.0×10^7 CFU/g; *Saccharomyces cerevisae*, 4.3×10^6 CFU/g; *Bacillus subtilis* 2.0×10^6 CFU/g). Some researchers have reported that *Bacilli* and *Lactobacilli* bacteria alone or in combination can enhance humoral and cell-mediated immune responses (European Food Safety Authority, 2010) as well as further promote anti-bacterial and anti-viral activities. The protective effects of feeding immune-enhancing probiotics can reduce the severity of *E. coli* infection, and this reduction may be associated with enhanced humoral and cellular immune responses (Shu and Gill, 2002). Probiotics also enhance the systematic antibody response to soluble antigens in the serum and participate in the development of immunity (Christensen et al., 2002).

The poorest level of immunoglobulins in the antibiotics-treated group may be due to the immunosuppressive action of aminoglycoside, which has been reported to reduce the production of antibodies after enteric challenge (Roura et al., 1992). We found reduced serum TNF-α concentrations in both the antibiotics - and probiotics-supplemented groups. Our result are consistent with Isolauri et al. (2001), who reported that probiotics mediate the suppression of lymphocyte proliferation and cytokine production by T cells, thereby down regulating the expression of proinflammatory cytokines such as tumor necrosis factor-α (Stewart et al., 1996).

Conclusion

In total, the findings of the present study indicate that dietary supplementation of *Lactobacillus*-based probiotics positively affected body weight gain and feed intake, whereas feed conversion ratio was improved by *Bacillus*-based probiotics. Both probiotics positively altered the microbial environment. Moreover, *Bacillus*-based probiotics increased serum IgG production. Considering these results, we suggest further feeding trials in order to better understand the effects of such additives as antibiotic alternatives as well as to elucidate their mechanisms of action underlying immunity enhancement in weaned piglets.

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Full Length Research Paper

Antibiotic resistance pattern of methicillin-resistance and coagulase-negative *Staphylococcus* isolates among hospitalized patients at a tertiary hospital in Gansu, North-western China

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This study aimed at determining the prevalence and antibiotic resistance pattern of methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin resistant coagulase negative staphylocci (MRCoNS) isolated from various clinical specimens obtained from hospitalized patients in a Tertiary Hospital in Gansu, North-western, China from 2008-2011. Bacterial isolates were identified morphologically and biochemically using the standard laboratory operation procedures. A total of 1002 isolates were obtained from 986 clinical specimens. The frequency of MRSA 690 (68.9%) isolates were identified as *Staphylococcus aureus* and 312 (31.1%) were coagulase negative *staphylococci.* The frequency of MRSA by the Cefoxitin disk diffusion test was 56.7% (391/690) and 73.3% (229/312) were MRCoNS. The rate of multidrug resistance observed was 61.8% (242 /391) for MRSA and 71.2% (163 /229) for MRCoNS. The antibiotc susceptibility test was determined by Kirby-Bauer disc diffusion method with zones of inhibition evaluated according to the latest Clinical and Laboratory Standard Institute (CLSI) guidelines recommendations. Mueller-Hinton agar was utilized for antimicrobial susceptibility testing. All MRSA and MRCoNS were 100% resistant to penicillin and oxacilin, MRSA isolates showed high resistance to erythromycin (98.7%), clidamycin (93.1%), and ciprofloxacin (89.2%) as compared to other drugs while MRCoNS isolates showed high resistance to erythromycin (96.7%), trimetoprim/sulphametoxazole (86.7%), and clidamycin (72.4%). However, all MRSA and MRCoNS isolates were sensitive to vancomycin, Linezolid and Teicoplanin. These finding indicate that MRSA and MRCoNS were common infections among hospitalized patients at Gansu Provincial People's Hospital (GPPH). The isolates showed high level of resistance to routinely used antimicrobial agents. This calls for strict antibiotic policy, continuous monitoring of antibiotic susceptibility pattern of all *S. aureus* and coagulase negative staphylococci. Further molecular study on MRSA epidemiology in future is desirable, to know the mechanism of resistance, find new antimicrobial agents and study the treatment strategies.

Key words: Antibiotic susceptibility pattern, methicillin resistant *Staphylococcus aureus* (MRSA), methicillin resistant coagulase negative staphylocci (MRCoNS), multidrug resistance.

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin resistant coagulase negative staphylocci (MRCoNS) are important nosocomial pathogen which evolved shortly after the introduction of methicillin, nafcillin and oxacillin antibiotics and were first reported in the United Kingdom in 1961 (Enright et al.,.. 2002;

Cookson et al., 2003). These pathogens account for a serious public health problem worldwide and have been widely implicated in skin and soft tissue infections, ventilator associated pneumonia, catheter associated bacteraemia (Ansari et al., 2012), and many other infections among hospitalized patients (Ansari et al., 2012).

Methicillin resistance (presence of the *MecA* gene responsible for methicillin resistance) is a predictor of resistance to all antibiotics belonging to the beta-lactam family (Rohrer et al., 2003). Infections due to MRSA and MRCoNS are of special concern since they are always associated with prolonged hospital stay and increased cost of treatment. Also, infections with MRSA and MRCoNS are associated with higher mortality compared to MSSA and MSCoNS due to multidrug resistance. There have been reports of higher incidence of bacteraemia and septic shock among the MRSA and MRCoNS infected patients and mortality has also been directly related to peumonia among patients with these infections (Shi et al., 2011).

This study aimed at determining the prevalence of MRSA and MRCoNS infection among hospitalized patients at Gansu Provincial People's Hospital (GPPH), a tertiary hospital in North-western China, and to examine the resistance pattern of these strains to antibiotic commonly used to treat MRSA and MRCoNS infection for the purpose of generating antimicrobial policy.

MATERIALS AND METHODS

Isolates and identification of clinical specimens

A total of 1002 *Staphylococcus* isolates were recovered from hospitalized patients at GPPH, north-western, China, between January 2008 to December 2011. The study was carried out in the clinical microbiology laboratory at GPPH. Specimens from which *Staphylococcus* were isolated included wound swabs, pus/aspirates (from sites other than wound), sputum, bronchoalveolar lavage fluid, throat swabs, blood and urine,and these were submitted to the laboratory as part of routine specimens. Specimens were cultured on Oxacillin Resistance Screening Agar base (ORSAB, OXOID, UK) for 24-48 h at 35°C, and then the plates were examined. The medium uses aniline blue to demonstrate mannitol fermentation in staphylococci. The dual antibiotic supplement (oxacillin, 2.0 µg/ml; polymixin B, 50,000 IU/l) and the presence of 5.5% NaCl have the potential to reduce the growth of nonstaphylococcal organisms and to select for the growth of MRSA. Isolates were subsequently identified by conventional tests, including Gram staining and tests for catalase activity, tube coagulase activity, latex agglutination (Biomerieux, Marcy L'Etoile, France), and morphologically and biochemically by the standard laboratory methods (Baird, 1996).

Antibiotic susceptibility testing

Antibiotic susceptibility test was performed for the following drugs: penicillin (10U, Oxoid, UK), oxacillin(1 ug), ciprofloxacin (5 µg, Oxoid, UK), erythromycin (15 µg, Oxoid, UK), clindamycin (2 µg, Oxoid, UK), trimetoprim/sulphametoxazole (1.25/23.75 µg, Oxoid, UK), and vancomycin (30 µg, Oxoid, UK); linezolid. Kirby-Bauer disc diffusion technique was used in which staphylococcus isolates were inoculated onto the surface of Mueller-Hinton agar (MHA) plate (Oxoid, UK), before antibiotic discs were laid on the surface. The plates were incubated overnight at 35°C. The zones of inhibition were evaluated according to the Clinical and Laboratory Standard Institute (CLSI, 2011) guidelines.

The vancomycin e-test was performed as per manufacturer's instructions. An elliptical zone of inhibition was obtained after incubation and MIC was read where ellipse intersected the strip. *S. aureus*, ATCC 29213 and *E. faecalis*, ATCC 29212, were used as vancomycin susceptible controls and *E. faecalis* 51299 as vancomycin resistant control. Readings were taken as per the guidelines of CLSI.

MRSA and MRCoNS conforming test

Methiciliin-resistance was determined by using Cefoxitin Disk (10 µg, Oxoid, UK) Diffusion Test; MRSA isolates with zones of inhibition around cefoxitin disc less than or equal to 21mm diameter; But MRCoNS diameter was 24 mm (CLSI, 2006). *S. aureus* ATCC 29213 was used as methicillin-sensitive control strain while *S. aureus* ATCC 43300 was used as methicillin-resistant control strain. Data entry and processing was done using computer software WHONET version 5.6.

RESULTS

1002 Isolates were obtained from various clinical specimens from hospitalized patients out of which 690(68.9%) isolates were identified as *S. aureus;* and 312(31.1%) were coagulase negative *staphylococci*. Among all isolates, the frequency of MRSA by the Cefoxitin disk diffusion test was 56.7% (391/690) and 73.3% (229/312) were MRCoNS. The rate of multidrug resistance observed was 62% (242 /391) for MRSA and 71% (163 /229) for MRCoNS. The highest rate of isolation of MRSA was from burn wound swabs (212 = 54.2%), followed closely by Sputum (92 = 23.5%), and pus/aspirates (36 = 9.2%); MRCoNS strains were isolated from burn wound swabs (80=34.9%), urine specimens (64=27.9%) and pus/aspirates (38=16.6%).

The antibiotic resistance and susceptible rate of all isolated *S. aureus* strains is as shown in Table 1.

All MRSA and MRCoNS strains were resistant to penicillin and oxacillin; MRSA and MRCoNS also showed greater resistance compared to MSSA and MSCoNS. MRSA had higher resistance rates to erythromycin (98.7%), clindamycin (93.1%), ciprofloxacin (89.2%) and rifampin (72.8%), while resistance rates of MRCoNS to the above drugs were 96.7, 56.7, and 72.4% respectively. MRSA and MRCoNS were susceptible to vancomycin, linezolid and teicoplanin.

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Abbreviations: CoNs, Coagulase negative staphylococcus; **MRSA,** methicillin resistant *Staphylococcus aureus*; **MRcoNS,** methicillin resistant coagulase negative staphylococci.

Table 1. Resistance and susceptible rate of MSSA, MRSA and MSCoNS, MRCoNS.

Antibiotic	MSSA		MRSA		MSCoNS		MRCoNS	
	Resistance rate (%)	Susceptible rate (%)	Resistance rate (%)	Susceptible rate (%)	Resistance rate (%)	Susceptible rate (%)	Resistance rate (%)	Susceptible rate (%)
Penicillin	93.9	6.1	100.0	0	58.1	41.9	100.0	0
Oxacillin	0	100.0	100.0	0	0	100.0	100.0	0
Rifampin	2.1	97.9	72.8	27.2	0	100.0	6.7	93.3
Ciprofloxacin	5.6	94.4	89.2	10.8	0	100.0	56.7	43.3
Erythromycin	76.1	23.9	98.7	1.3	86.7	13.3	96.7	3.3
Trimetoprim/ sulphametoxazole	1.9	98.1	19.0	81.0	29.6	70.4	86.7	13.3
Clindamycin	43.6	56.4	93.1	6.9	29.8	70.2	72.4	27.6
Vancomycin	0	100.0	0	100.0	0	100.0	0	100.0
Linezolid	0	100.0	0	100.0	0	100.0	0	100.0
Teicoplanin	0	100.0	0	100.0	0	100.0	0	100.0

DISCUSSION

MRSA and MRCoNS are recognized as a major cause of nosocomial infections which result in significant morbidity and mortality (Schumacher-Perdreau, 1991).

In our study, 56.7% strains of MRSA and 73.3% MRCoNS were detected from 2008-2011. Similar high positive rate have been reported from different regions in China (Wang et al., 2008); the high MRSA infection rate in this study might not be unconnected to the poor infection control program in our hospital with no or poorly documented antibiotic policy.

This study, like previous studies, had demonstrated that MRSA are more resistant to various group of antibiotics compared to MSSA. It is not surprising that all the MRSA tested in this study were 100% resistant to penicillin whereas 93.9% of MSSA were resistant to the antibiotic; the finding only substantiates the fact that resistance to methicillin predicts resistance to other beta-lactam drugs. All *S. aureus* (both MRSA and MSSA) in this work were sensitive to vancomycin, linezolid and, teicoplanin. This finding is similar to those of some previous studies. The MRSA were highly resistant to erythromycin (98.7%), and the high rate (63.3%) of multi-drug resistant MRSA (resistance to three or more families of antibiotic at a given point in time), with up to 42.9% of them being resistant to more than three non-vancomycin antibiotic families, found in this study is worrisome considering the ability of *S. aureus* to spread easily by direct or indirect person-to-person contact with resultant therapeutic difficulties. Vancomycin linezolid and teicoplanin were the three antibiotics with 0% resistance even with multi-drug resistant strains and thus remain the best therapeutic option in our setting, however, the drug is widely unavailable and other available therapeutic options must be considered. Trimetoprim/sulphametoxazole is favored considering the lower rate of resistance (19.0%) to it by MRSA in this study. The MSSA are generally still of lower resistant rate (compared to MRSA) to commonly used antistaphylococcal agents tested in this study, however, ciprofloxacin (5.6%), and probably Trimetoprim/ sulphametoxazole (1.9%) and rifampin (2.1%) were good enough to be considered for management of infections due to these MSSA.

Conclusion

MRSA and MRCoNS are prevalent in GPPH, China. Our study demonstrates that the sensitivity of MRSA and MRCoNS to vancomycin, linezolid and teicoplanin are 100% which further emphasizes that it is still the drug of choice for MRSA and MRCoNS infections. In our study, about 81.0% of the MRSA and 13.3% MRCoNs were susceptible to trimetoprim/sulphametoxazole.

Therefore, trimetoprim/sulphametoxazole has an important role in the management of MRSA or MRCoNS infections. The MRCoNs showed very high resistance for trimetoprim/sulphametoxazole (86.7%) and erythromycin (96.7%). We recommend that frequent monitoring of susceptibility patterns of MRSA and MRCONS and the formulation of a definite antibiotic policy may be helpful in decreasing the incidence of MRSA and MRCONS infection.

The study had demonstrated a high prevalence rate of MRSA with high rate of resistance to commonly used anti-staphylococcal agents. A large proportion of these MRSA and MRCoNS were found to be multi-drug resistant. There is need for continuous monitoring of antibiotic susceptibility pattern of all *S. aureus* isolates for selection of appropriate therapy. Also, infection control measures such as handwashing and other aseptic techniques must be followed to avoid therapeutic difficulties associated with these resistant pathogens. Further, molecular studies for studying and monitoring the epidemiology of MRSA and the multi-drug resistant MRSA in future is highly desirable. In developing countries, knowledge of antimicrobial resistance patterns is essential to define empirical therapy.

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Microbiological comparison of microwave and traditional thawing processes for poultry meat

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Health agencies official has been convinced of the efficiency of microwave based process mainly due to its bactericidal property and safety (non-ionizing radiation). However, many biotechnological aspects of this new method are still unknown and have raised safety concerns. This study aimed to validate the application of the thawing process of a large poultry meat block under high frequency microwave oven (2.45 Ghz within 60 s). Compared to the traditional thawing method (4°C for 48 h), we combined a visual observation and bacteriological analysis. The microwave thawing process was clearly faster than the conventional process, by at least three times. Both microwaved samples and control samples complied with the standard requirements, even after five weeks of microbial-screening of total mesophilic flora, fecal coliforms, sulfite-reducing anaerobic, *Staphylococcus aureus* and *Salmonella*. In the exposure to microwave, there was also less loss of water compared to the traditional method (2.33±0.21 l < 3.45±0.21 l). Although it showed overheating signs due to the high frequency used, it did not compromise the shelf life of the product. We validated the microbial-safety of the microwave thawing process; however, adjusting the frequency would result in better quality output and alleviate side effects in the frozen poultry.

Key words: Thawing process, poultry meat, chicken, microwave oven, quality management.

INTRODUCTION

Microwave (MW) irradiation has become increasingly popular in food processing, to brown, dry, cook and particularly in improving food quality and shelf life, despite the lack of official technical criterion (Yahmaee and Durance, 2005; FDA, 2006; *Codex Alimentarus*, 2007; Taher and Farid, 2011; Lin et al., 2014).

This technology is used mainly in household applications and recently in food industries (Ohlsson, 1989; Chaum et al., 2009, Vadivambal and Jays, 2007). In fact, its ability to interfere with biological systems is gaining popularity, particularly its heat production dynamic. In fact, MW induces heat by two reactions; the first is dipolar polarization, the cause of rotation and vibration of dipoles like free water (a_w). The second is a complementary reaction due to ionic conduction of free charges stimulated by electromagnetic field. The two reactions are triggered by the MW radiation and induce internal fractions within the foodstuff, resulting in heat production. The ionic density and moist content of a given stuff both shape the ability of the stuff to couple with MW, namely, permittivity or dielectric characteristic of the matter (Heddleson and Doors, 1994; Ang et al., 1977; Chandrasekaran et al., 2013; Venkatek and Raghavan, 2004; Komarov et al., 2005; Thostenson and Chou, 1999).

The selective MW heating effect seems to be valuable, especially in food processing and food decontamination

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(Ohlsson and Bengston, 1975; Sivaramakrishnan, 2010; Tyagi and Lo, 2013). For this purpose, it has been introduced as a reliable and efficient technology, to reduce microbial hazards and produce safer products (Aranzana et al., 2013; Tinoco et al., 2014; Farkas, 1998; Thayer, 1995). Consequently, many good studies have been done on the biotechnological aspects of microwave, and have succeeded in developing promising aspects of pasteurization and even sterilization. (Holsson and Bengtsson, 1975 ; Chandrasekaran et al., 2013; Byrne et al., 2010; Dov et al., 2013, Zhang et al., 2010).

For instance, Lau and Tang (2002) have identified a fast decontamination effect in asparagus within a short time exposure; they establish recommendation on the use of MW for sensitive foodstuffs. Admittedly, MW heating process requires less thermal exposure, to reach the safety scales (Tinoco et al., 2014); therefore, it leads to better nutriments preservation (Koné et al., 2013). As a result, it has been affiliated to the so called group of “Cold pasteurization technologies.” However, the lethal effect of MW has been subjected to controversies, and seems to be more complex than expected (Wu and Yao, 2011; Hamoud-Agha et al., 2013). Put in this way, many authors have done deep investigations on MW effects. Papadopoulou et al. (1995) first reported a possibility of differences between thermal and electromagnetic effects on microorganisms. As the two effects seem to be mitigated and closely linked (Byrne et al., 2010), many trials have focused on each effect discreetly and established a major molecular modification (Nasri et al., 2013; Shames et al., 2008; Tinoco et al., 2014).

Actually and since the mid-sixties, poultry industry has been strongly supported by the authority, and has known a great improvement. Currently, poultry meat represents over 25% of the livestock sector in Tunisia and provides 53% of all meats. The production of poultry meat reached its saturation level in 2012, about 250 thousand ton with a value of 152 millions of US dollar (Tunisian Ministry of Agriculture, 2013). To balance the commercial flow, authorities regularly freeze the extra stocks, in particular, during the holy month, when demand of Tunisian customers reaches a peak and fresh meat is almost scarce.

In that connection, we have proposed to evaluate the effects of high frequency of MW on the quality of the poultry meat during the industrial thawing process. Thereby, we compared that process to the traditional thawing method without MW. And as a first published study on microwaved frozen chicken in Tunisia, we formulated general recommendations on the use of MW radiation in food processing as a contribution to the quantitative food risk assessment and HACCP plans being implemented all over the Agro-industry.

MATERIALS AND METHODS

Material for thawing

We used a high frequency microwave oven (2.45 Ghz) "Ferrite®" with a wavelength of 12.2 cm. The oven can be loaded up to 25 kg and is provided with an electronic scale. For manipulation, we used large sterile plastic bags, designed for single use. A digital thermometer was used for food, with a measurement capacity from - 20±0.1°C to + 150±0.1°C. Its probe was a stainless steel of 12.5 cm length.

Equipment for sampling

Sampling equipment was sterile of high purity and had no influence on the microflora of the product. The sampler wore a gown, cap, mask and gloves, equipped with bags, labels and a cooler thermal insulation. Plastic bags were sterile and with an appropriate size for sampling.

Colony counting apparatus

The colony counting apparatus was equipped by a lighting system with a black background, and provided with a magnifying glass of 1.5, and an electronic counter. Incubators maintained inoculated media, boxes and bottles within a temperature range of 30 to 46°C.

Thawing chicken

Thawing in cold positive

20 kg of whole frozen chickens (18 and 20 chicken carcasses) were maintained in clean plastic yellow boxes at a maximum of -18°C. In each box, a single used plastic wrap was introduced. Then, we placed five to six chickens on the back arranged in a single level per box.

Boxes were loaded into the stock room, with temperature between 1 and 3°C, not exceeding 4°C. An empty red box was put below all boxes containing the product, without touching the ground directly. Then, the thawing processes were triggered and these lasted for 48 h.

Thawing in the microwave

We conducted the thawing in the microwave oven as it was carried out in cold storage. Once the boxes were loaded, they were placed one by one in the cavity of the microwave oven. The radiation cycle lasted for 60 s with a power of 20 KW; the energy dispatched into the foodstuff was 60 KGy.

On leaving the microwave oven, chickens were kept in a temperature between 4 to 2°C. Subsequently, the boxes were stacked one upon the other, with an empty red box below; after which they were returned to the stock room with maximum temperature equal to 4°C to complete the thawing for 15 hours.

We weighted the global load of the chicken before and after each process. The wasted water was collected in graduated bottle and evaluated at the end of the experiments; consequently, weight losses were estimated.

Sampling and sample transport

Immediately, at the end of the thawing processes, fives simples by batch and tow bath process were pulled out from the microwaved and control group, from the top and lowest boxes, weekly. We fully complied with the conditions of the sampling, randomly and aseptically, following the recommendations of the Codex Standard (*Codex Alimentarius,* 2004). We majored the temperature of each simple before we packaged, saddled, labeled and saved it in a cold

Table 1. Results of microbiological analysis obtained from the **top boxes** of chicken thawed by slow process.

Flora	Viable cell count (log_{10} CFU/g)				
	Week 1	**Week 2**	**Week 3**	**Week 4**	**Week 5**
TMF	3±0.5*	2.77±0.5*	3±0.5*	2.47±0.5*	1±0.5*
FC	<1±0.7*	<1±0.7*	<1±0.7*	<1±0.7*	<1±0.7*
SRA	<1±0.6*	<1±0.6*	<1±0.6*	<1±0.6*	<1±0.6*
SA	<1.69±1*	<1.69±1*	<1.69±1*	<1.69±1*	<1.69±1*
Salmonella	Absent	Absent	Absent	Absent	Absent

n = 5; *Error signifiance ; TMC, total mesophilic flora ; FC, fecal coliforms; SRA, sulfite-reducing anearobic; SA, *Staphylococcus aureus*.

Table 2. Results of microbiological analysis obtained from the top boxes of chicken thawed by rapid process.

Flora	Viable cell count (log_{10} CFU/g)				
	Week 1	**Week 2**	**Week 3**	**Week 4**	**Week 5**
TMF	2±0.5*	2.3±0.5*	2±0.5*	2±0.5*	1±0.5*
F C	<1±0.7*	<1±0.7*	<1±0.7*	<1±0.7*	<1±0.7*
SRA	<10±0.6*	<1±0.6*	<1±0.6*	<1±0.6*	<1±0.6*
S. A	< 1.69±1*	< 1.69±1*	< 1.69±1*	< 1.69±1*	< 1.69±1*
Salmonella	Absent	Absent	Absent	Absent	Absent

n = 5; *Error significance; TMC, total mesophilic flora ; FC, fecal coliforms; SRA, sulfite-reducing anearobic; SA, *Staphylococcus aureus*.

container, until the time of shipment to the laboratory (within 12 hours).

Preparation of test samples

Upon arriving at the Military Laboratory of Food Analysis, chickens were spread out in a stainless steel and 10 g of mixed portions were collected aseptically (7 g of flesh and 3 g of skin). The sample was placed in a sterile stomacher bag, in which we added 90 ml of buffered peptone water. The whole was milled using a stomacher for 2 to 3 min. The obtained homogenate was 10^{-1}; from this original solution, we conducted a series of decimal dilutions, up to 10^{-5}.

To search for *Salmonella* spp., 25 g of the skin of the chicken was suddenly taken, to which we added 225 ml of buffered peptone water. The performed essays followed the recommendations of the order of December 21th, 1979 (JORF, 1980).

Bacteriological analysis

The enumeration of Total Mesophilic Flora (TMF) was done according to the International Standard, ISO 2293 (ISO, 1976), by counting the observed microorganisms at 30°C. The enumeration of Fecal Coliforms (FC) followed the French standard, NF V08-060 (AFNOR, 2009a), in which Thermotolerant Coliforms (TC) were evaluated by counting the observed colonies at 44°C. The evaluation of *Staphylococcus aureus* (SA) was made according to ISO 6888-1 (ISO, 1999). A special interest was given to the coagulase positive staphylococci by using a specific medium (Baird-Parker solid medium) at 37°C. Sulfite-Reducing Anaerobic (SRA) culture was carried out according to the French Standard NF V08-061 (AFNOR, 2009b) at a temperature of 46°C. The detection of *Salmonella* was performed according to ISO 6579 (ISO, 2002). Incertitude errors were evaluated for each of the bacteria separately, except for *salmonella,* where no reliable method of calculation is currently available. In fact, the error significance was estimated by computing the standard derivation of the reproducibility, according to the ISO Standard 19036 (ISO, 2006), recommended by the General Directorate of Health and Consumer (European Commission, 2006). We estimated the error rates from 0.5 to 1 log_{10} reduction.

RESULTS AND DISCUSSION

Food safety impact

In our study, we demonstrated that, bacteria are usually related to quality (TMF, FC and SRA) and thus reflecting food safety concern (SA and *Salmonella*) meet its standards and satisfactory achieve the safety requirements in both upper (Tables 1 and 2) and lowest cases (Tables 3 and 4). Indeed, for the TMF, the seeds level obtained were well below the threshold of 5 log_{10} CFU/g. Also, the results of FC were below the required threshold of 3 log_{10}, followed by 1 log_{10} CFU/g.

The count of SRA throughout five weeks was less than 1 log_{10} CFU/g, and below the value set at 1.47 log_{10} CFU/g. In the same way, the results of SA were less than 1.69 log_{10} CFU/g, and met the safety standards (< 2.69 log_{10} CFU/g); thence it clearly averted toxin formation. We also noted that all tests are free of *Salmonella* and would not harm consumer's health.

Table 3. Results of microbiological analysis obtained from the bottom most boxes of chicken thawed by slow process.

Flora	Viable cell count (Log_{10} CFU/g)				
	Week 1	**Week 2**	**Week 3**	**Week 4**	**Week 5**
TMF	1±0.5*	2.69±0.5*	3±0.5*	2±0.5*	1±0.5*
Fecal coliforms	<1±0.5*	<1±0.7*	<1±0.7*	<1±0.7*	<1±0.7*
SRA	<1±0.6*	<1±0.6*	<1±0.6*	<1±0.6*	<1±0.6*
S. aureus	< 1.69±1*	< 1.69±1*	< 1.69±1*	< 1.69±1*	< 1.69±1*
Salmonella	Absent	Absent	Absent	Absent	Absent

n = 5; *Error significance; TMC, total mesophilic flora; FC, fecal coliforms; SRA, sulfite-reducing anearobic; SA, *Staphylococcus aureus*.

Table 4. Results of microbiological analysis obtained from the bottom most boxes of chicken thawed by rapid process.

Flora	Viable cell count (Log_{10} CFU/g)				
	Week 1	**Week 2**	**Week 3**	**Week 4**	**Week 5**
TMF	1±0.5*	2±0.5*	2.3±0.5*	2±0.5*	1±0.5*
Fecal coliforms	<1±0.7*	<1±0.7*	<1±0.7*	<1±0.7*	<1±0.7*
SRA	<1±0.6*	<1±0.6*	<1±0.6*	<1±0.6*	<1±0.6*
S. aureus	<1.69±1*	<1.69±1*	<1.69±1*	<1.69±1*	<1.69±1*
Salmonella	Absent	Absent	Absent	Absent	Absent

n = 5; *Error significance; TMC, total mesophilic flora; FC, fecal coliforms; SRA, sulfite-reducing anearobic; SA, *Staphylococcus aureus*.

Moreover, the obtained averages of TMF under the two processes imply that the upper boxes are slightly loaded with germs than the lowest boxes. The monitored rates were 2.44 log_{10} > 1.93 log_{10} CFU/g (Tables 1 and 3) and 1.86 log_{10} > 1.66 log_{10} CFU/g (Tables 2 and 4) subsequently for slow and rapid processes. Meanwhile, we did not observe a change in the contamination distribution after exposure to MW.

In the light of the foregoing results, we can conclude that, bacteria were not fully eradicated during the MW irradiation. This matches the results of Schlissemberg et al. (2013), who did not succeed in inactivating all the aerobic mesophilic bacteria experimentally inoculated onto ground beef, after exposing to radio frequency of 1000 MHz during 7.5 min. To illustrate this point, scientists (Harisson and Carpenter., 1989) described an effective proliferation of radiotolerant and thermotolerant bacteria even after prolonged exposure to MW. They have considered *Lesteria* as the most resistant vegetative bacterium, and at present, it is being introduced as a safety indicator of food processing (Jo et al., 2007, Kamat et al., Nair, 1996, Schlisselberg et al., 2013, JORF, 2007).

These findings are corroborated by the absence in our study of significant differences of microbial load, between microwaved food and the control group. We also underscore that exposure to MW kept the same bacterial distribution between both lowest and top boxes (p-value >0.1), using analysis of variances test (ANOVA) of Biostat TGV ® software Jussieu, Paris.

In fact, the thermal destructive point within the samples was not reached; the whole temperature monitored did not exceed 4°C, which may explain the persistence of some bacteria after MW of exposure. These findings are expected, since many reports scientifically demonstrated the absence of the destructive effect of MW under a minimum required exposure. That is, we have not been able to confirm the inactivation effect of the high MW frequency at cold temperature, in spite of the high energy (60 KGy) deployed in this case.

At this particular point, our results joined the conclusions of Welt et al. (1994), which did not find any inactivation effect against the spores of *Clostridium sporogenes* at sublethal temperature, even after the use of high power (400 W). According to Lu et al. (2011), the decontamination of *Salmonella enteritica,* experimentally inoculated onto tomatoes, was 1.45 log_{10} reductions after 40 s exposure to MW field of 700 W; the maximum monitored temperature was 48°C.

Clearly, Lau and Tang (2002) had more success in inactivating *Salmonella*, within few dozen of seconds, in both Japanese pepper and coriander. The authors had achieved a destruction rate of 5 log_{10} reductions, following exposure to MW field of 950 W; they also pointed out the need to reach a lethal temperature within the sample (63°C) to observe a significant bacterial des-

truction. Correspondingly, Gentrya and Roberts (2005) consider that MW would effectively diminish *Echerichia coli* contamination in apple cider by 5 $\log_{10}$ reductions, using a continuous flow exposure system, with a power of 200 W and a temperature of 40°C.

The same conclusions were drawn in meat processing. In particular, Apostolo et al. (2005) achieved a high level of decontamination, as high as 6 $\log_{10}$ reductions, of *E. coli* in chicken, by applying a power of 800 W for 30 s; the temperature of the food was 75°C.

The same attempt was reproduced on milk, with comparable inactivation amount in both *Lesteria innocula* and *E. coli;* the reductions were 5 $\log_{10}$ and 6 $\log_{10}$ respectively. The power scale formerly used was slightly above the earlier one (1200 W during 55 s), with less heat production (65°C) (Awua et al., 2005). Besides, MW was found to control *Salmonella* in eggs 5 times faster than the conventional heating method and then provides an effective pasteurization scale (61.1°C within 2.5 min) (Dev et al., 2008; Sivaramakrishnan, 2010) .

According to Byrne et al. (2010), sterilization was also effective in meat. The authors had decreased the amount of *Bacillus cereus* spores by 1.8 $\log_{10}$ and *Clostridium perfrengins* spores by 4.1 $\log_{10}$ reductions, by applying a radiofrequency of 500 W combined to hot water (80°C).

In other studies, MW radiation had no effect on pathogen microbe in chicken meat; in fact, despite, the higher MW scale used in chicken (1138.8 W during 30 s), the survival abilities of *E. coli* and *Campylobacter jejuni* were not eroded. In that case, the temperature in the meat did not exceed 47°C (Goksoy et al., 2000), far from the safe level, namely 74°C (*Codex Alimentarus*, 1993). Our results are in agreement with the authors' observation; they further confirmed heat dependant inactivation of MW.

Similarly, Palaniappan et al. (1990) believed that the inactivation effect was only due to the ohmic heating of the radiations. Others reported deadly cellular and genetic injuries after a sublethal exposure to high MW frequencies (Nasri et al., 2013; Shamis et al., 2008; Uemura and Isode, 2002). Hong et al. (2004) and Wu and Yaho, (2011) placed emphasis on the interference of MW with critical cell compounds, as well as DNA, ARN and Cell membrane. They established the occurrence of multiple disruptions and mutations, just like Dreyfuss and Chipley (1980), who support that MW inactivation cannot be solely correlated to the thermal effect, but that many enzymatic alterations may explain the electroporation and metabolic breakdown of bacterial cell at mild temperatures.

In the following earlier mentioned results, it appears that MW irradiation did not change the contamination patterns, neither in the short run (just after the exposure) nor in the long run (five weeks after the exposure). This implies that the persistent bacteria have kept their capacity to survive and multiply, regardless the process of thawing used in chicken*, inter alia;* no deadly athermal (MW) effects were proved at our industrial level (p-value > 0.1), using analysis of variances test (ANOVA) of Biostat TGV ® software.

Hence, we admit that the inactivation effect of MW is an energy dependent phenomenon, in total approval with most reports (Apostolo et al., 2004; Awua et al., 2005; Lau and Tang et al., 2002; Lu et al., 2011; Dreyfuss and Chipley, 1980; Heddleson and Doores, 1994; Harrison, 1988). In that case, to be effective, the MW energy absorbed by the foodstuff must certainly be converted to a minimum heat threshold (Goksoy et al., 2000). Otherwise, the bacteriolethal temperature would not be reached.

Last but not the least, the variable anisotropic character of the frozen food and its dynamic boundary conditions complicate even more MW behavior, and especially its impact on food quality (Ang et al., 1977; Rattanadecho, 2004).

Impact on food quality

Bengtsson and Risman (1971) and Akkari et al. (2006) studied the dielectric properties of food and water at different temperature ranges, in an attempt to understand the MW dynamics. They stated that frozen food has less permittivity compared to fresh food, and is less able to absorb and convert (MW) energy into heat. Admittedly, the dielectric property is meanly correlated with moist content, in as much as free water (a_w) has a high permittivity and improves the energy absorption of a given stuff (Chandrasekaran et al., 2013; Akkari et al., 2006; Ryyniinen, 1995).

In fact, during the thawing of the ice, the dietetical property of the frozen food would go in two different ways, depending on the (a_w) amount. While the permittivity of the liquid phase is increasing along with the melting ice, conversely the permittivity of the frozen part remains low and behaves as transparent materiel to MW radiations (Chaiyo and Rattanadecho, 2011). As the tow opposite reactions are going on, further heat runaway is induced, with substantial thermal variation within the matter (Yang and Gunasekaran, 2004; Venkatesh and Raghavan, 2004, Chaiyo and Rattanadech, 2011). The final result is an incomplete thawing with loss of water (Ryymine, 1995; Komarov et al. 2005, Taher and Farid, 2001).

In our study, we also met the same drawbacks of partly thawing reaction with water losses, but we have succeeded in alleviating it, by putting the meat at rest at 4°C until the establishment of the thermal equalization between the two phases of the foodstuff.

With time, water losses following the traditional thawing method were more compared to the MW thawing process (3.45±0.21 l > 2.33 ±0.21 l). Likewise, Taher and Farid (2001) have mathematically validated those observations by simulating the MW dynamic in frozen meat. We also

estimate that, water losses have influenced the final weight of the meat blocks, which were 17.5 ±0.15 Kg at the end of the MW thawing process and 16.37±0.15 Kg at the end of the traditional thawing method, with corresponding weight reduction of 12.5 and 18.15%, respectively. The results were statistically significant ($p < 0.05$) using t-student of Biostat TGV ® software.

The observations of Schlisselberg et al. (2013) meet our finding and support the occurrence of weight loss. Indeed, the authors found 17% of weigh loss in fresh ground beef after exposure to radio frequency for longer period (7.5 min).

Actually, after the use of the MW radiation, the thermal balance was reached faster, by at least three times, than in the conventional process; in fact the thawing lasted for15 hours during the MW processing; whereas the traditional one took 48 h. Undoubtedly, our observations met the conclusion of most specialists in this field (Tinoco et al., 2014; Schlisselberg et al., 2013; Dev et al., 2008), which confirmed the fast MW processing of foodstuff, and the less thermal load requirement of MW heating (Koné et al., 2013; Lau and Tang, 2002).

In our experiment, we provided a first index of the microbiological safety and rapidity of MW in thawing poultry meat, under the specific condition of the thermal equalization period. Although, we have observed uneven cooking signs within the samples. In fact, the external aspect and the color uniformity of the chicken are important elements for consumers' acceptance. These criteria were depreciated by the appearance of red spots (more than two spots per chicken) located especially on the thighs. Actually, Goskoy et al. (2000) and Apostolou et al. (2004) explained that overheating marks (or hotspot effects) are a common drawback of the MW usage. They also asserted that the high frequencies of the MW diminish the wave penetration within the foodstuff and increase the hotspot distribution, especially, around the edges of the chicken. Also, Hmou-Agha et al., (2013) showed the uneven distribution of the hotspot, and their concentration in the asymmetric shapes, with uneven cooking signs (Chandrasekaran et al., 2013; Harrison et Carpenter, 1989). These conclusions are same with our results especially, due to the high frequency we used in our experiment (2.45 GHz).

We also found that this anomaly has no effect on the shelf life of the product; the chickens complied with safety standards, even throughout five weeks of cold storage; likewise the demonstrations of Goskoy et al. (2000). Further, Abu-Gyamfi et al. (2008) found an appropriate agreement between quality preservation and microbial control in ready-to-eat chicken, by applying an optimum radiation of 3 KGy; as a consequence, the shelf life of the product was extended to at least 14 days. Also Uemura et al. (2010) improved the quality of soybean derived food; tofu, made from flash-radiated soybean milk had the best breaking strength.

Almost all authors are convinced that MW process enhances food quality preservation and taste retention (Cocci, 2008). Adedeji et al. (2009) added that microwave at 6.7 w/g power density decreases significantly the oil uptake in chicken nuggets during deep-fat frying, and then improves its nutritional value. In concordance with the observations of Arocas et al. (2011), who confirmed the protective effects of MW on the retro-gradation of starch during the thawing of ready to eat sauces, similarly Hill, (1994) & Bedoui et al. (2011), and (Fiore et al., 2013) provided evidences of the preservation of food texture and vitamins content after exposure to MW. Meanwhile, the energy consumed and heat exposure were less than that in conventional cooking method by at least 30 p.100 (Wang et al, 2012, Schlisselberg et al., 2013).

Conversely, Ozkoc et al. (2009) refuted these results and mentioned fast bread staling after exposure to MW. This is similar to the findings of Lu et al, (2011), who reported a structural damage in tomatoes after 50 s of exposure to MW. Also, Liu et al. (2002) described an increasing redness after meat irradiation, due to modifications of myoglobin. These results were supported by Wu and Yao, (2011); Harrison et al. Carpenter (1989), who observed an increase of food microbial load after exposure to MW.

Many factors would explain this discrepancy over the authenticity of MW effects. Basically, the difficulty with MW processing is the large number of factors that rule the heat transfer behavior, which is inherent in food characteristics (thickness, size, shape, and permittivity) and not MW field proprieties (frequency, power, strength, wave length, and exposure period) (Harisson et Carpenter, 1989; Yang and Gusasekara., 2004; Lu et al., 2011; Vadivambal and Jayas, 2007). This may have influenced the results of this study.

In fact, previous reports agreed over the conditional and random reproducibility of microwaves effects (Goksoy et al., 2000; Law and Tang, 2002). Obviously, frequency use is admitted to have a leading role in MW efficacy and its penetration capacity within the food; it better averts hotspot effects (Goksoy et al., 2000; Venkatesh and Raghavan, 2004; Cherbanski et al., 2013). That is, pulsed MW heating with frequency sweeper has clearly proved its reliability in controlling bacterial contamination and averts uneven thermal distribution within the sample (Huang and Sites, 2007; Hung et al., 2006; Yang and Gunasekaran., 2004; Gentry et al., 2005; Kone et al., 2013; Vadivabal et al., 2010; Brow et al., 1999).

Also, Lau and Tang (2002) and Fiore et al. (2013) used a low microwave frequency to improve thermal distribution within food. Burfoot et al. (1988) too achieved the same performance using a frequency of 896 MHz, and effectively pasteurized ready to eat meat, whereas higher frequency (2450 MHz) induced less uniform thermal distribution.

Komarov et al. (2005) formulated the same assumption on frequency impact in thermal uniformity and proposed the use a frequency fare under the level that we have

already used, to enhance the ionic conduction and penetration of MW within foodstuff.

For the same purpose, Taher and Farid, (2001) suggested the use of a cyclic (MW) exposure, every 20 s, to avoid overheating effects in frozen food

Also, recent studies have confirmed the synergistic effect of combined strategies in controlling food hazard (Maktabi et al., 2011; Law and Tang, 2002, Adu-Gyamfi et al., 2008); in fact, water assisted (MW) heating, thawing or drying, which are currently being integrated in MW devices have increased the thermal sensitivity of bacteria (Byrne, 2010) and improved thermal uniformity within food (Miranda et al., 2012; Brody et al., 2012; Schlisselberg et al., 2013).

Thawing using air impingement technology has accelerated the thawing process, over four times faster than the conventional method, without increasing the temperature to a level, where microbial growth becomes a concern (Brent et al., 2006).

Conclusion and Recommendations

Microwave is already admitted to control food contamination and reduce nutriments destruction also. We have shown that microwaved food complies with the microbiological safety objectives and provides an appropriate level of security for the consumers. It is also a faster method to process food especially during crisis and food shortages, especially in Tunisia, where the freezing and thawing of the strategic stocks occur daily.

Although the validation of microbial safety of microwaved food is proved, it did not systematically imply the chemical stability of the processed stuff. In fact, a number of critical issues remain unresolved, which magnify the quality concerns and emphasize the need to use innovative technologies.

In future prospects, more studies are required to scrutinize the MW electrical property and adjust it to food characteristics

It is well established, that frozen food has poor permittivity and is deeply influenced by the temperature and frequency being used (Chaiyo and Rattanadech, 2011; Venkatesh and Raghavan, 2004; Ohlsson, 1989). For that reason, we suggest using a frequency sweeper for the thawing process, and to start with low frequencies and then increase it along with the progress of the melting point.

We also recommend considering the heating effect as the only effective element in bacterial inactivation, especially at industrial level to avoid any safety abuse that may imperil consumer's health.

At the legislative level, it is also necessary to enforce the current legislation and standardize the use of microwave, in order to establish accurate and repro-ducible technological performances and then better suit food safety and quality.

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