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

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Full Length Research Paper

Comparative biodegradation studies of cow dung modified epoxy with epoxy using an indigenously developed bacterial consortium

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Thermoplastic-based polymers and their blends are recalcitrant in nature. Based on their extensive use, huge amount of polymeric waste is being produced annually, which impart serious threat on the natural ecosystem. Considering this scenario, it is needed to take some immediate actions to keep the ecosystem dynamic and secure. Therefore, this study was carried out in order to evaluate an indigenously developed bacterial consortium for the biodegradation of epoxy and a blend of epoxy with cow dung that is cow dung modified epoxy (CME). These polymers were preliminary screened against the used bacteria individually for determination of optimum concentration to utilize them as carbon source. For this purpose, the comparative *in vitro* biodegradation studies were carried out using the bacterial consortium. Relatively, better biodegradation potential of developed consortium was observed for epoxy in comparison to CME, as higher biomass and more sustained growth of consortium was obtained in the presence of epoxy. Further, the progressive *in situ* degradation of epoxy and CME films was also conducted in the presence and absence of consortium for three and six months under natural conditions. For this purpose, bioformulation of bacteria was used to inoculate the soil. The treated samples were analyzed for their comparative spectral, thermal and morphological changes. Thus, the present study reveals that the used bacterial strains have the potential to act upon epoxy and CME polymers and capable to impart changes in the surface morphology during incubation in soil.

Key words: Epoxy, cow dung modified epoxy, biodegradation, bacterial consortium, Fourier transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM), thermogravimetry-differential thermal analysis (TG–DTG–DTA).

INTRODUCTION

The immense applications of synthetic polymers and their blends in different fields viz. packing, agriculture, marine, medicine, scientific, technological and household etc. has raised the issue of socio-environmental concern. Among

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Table 1. Bacteria potential for the biodegradation of Epoxy and its blends.

Polymer treated	Bacteria used	Reference
Stressed carbon-reinforced epoxy composite	Sulfate Reducing Bacteria (SRB)	Wanger et al., 1996
Epoxy and Epoxy Silicone Blends (ESBs)	*Psedomonas aeruginosa, Microbacterium sp, Bacterium Te68R and Psedomonas putida*	Negi et al., 2009
Epoxy and MF-modified polyurethane films	*Psedomonas aeruginosa strains*	Dutta et al., 2010
Organic epoxy	*Bacillus* and *Virgibacillus*	Wang et al., 2013
Epoxy resin	*Bacillus* and *Pseudomonas*	Pangallo et al., 2014
Epoxy-PU composites filled with ferrocene	Biological medium 199 (BM199)	Rudenchyk et al., 2015

Table 2. Isolation profile of the bacterial cultures used in the study.

Isolation site	Description of strain(s)	Reference
Artificial soil bed, Pantnagar	PN12	Satlewal (2008)
Soil sample, Pantnagar	MK3, MK4	Satlewal (2008)

these polymers, epoxy resins and its blends are of great scientific and technological interest due to their ease of processability and durability. Therefore, with the increase in production and exploitation of these polymers, the amount of waste has been raised enormously and accelerating the problem of solid waste disposal. Poor solid waste management in the developing countries causes major threat to public health and environment, and reduces the quality of life, in both urban and rural areas (Wang et al., 2011). Thus, need of this hour is to protect the environment by proper management of the polymeric waste using an ecofriendly biological means. The literature survey revealed that a variety of microbes are capable of carrying out biodegradation of epoxy and its bends (Table 1). Thus, microbial degradation may be one way to deal with the epoxy polymeric waste (Pangallo et al., 2014).

Furthermore, in a previous study, conducted by our research group, the rate of weight loss of epoxy and its silicon blend after 15 days of incubation under *in vitro* biodegradation using indigenously developed bacterial consortia was found to be 34.17 and 36.9%, respectively (Negi et al., 2009). Considering the fact, epoxy and a synthesized blend of Epoxy that is Cow Dung Modified Epoxy (CME) was tested in the present study for the biodegradation using the potential bacterial consortium. However, the ultimate aim of the study was to screen a cost-effective and eco-friendly additive (cow dung) to synthesize a blend with epoxy resin, which would be increasing the commercial applicability of the compound, after finding it's biodegradability. In addition, the growth profiling and potential of this used and other reported consortium was proved to be higher in comparison to a single strain (Sah et al., 2010; Pandey et al., 2015), thus, a bacterial consortium was used for comparative biodegradation assay.

MATERIALS AND METHODS

Starting materials

The epoxy and corresponding CME blend (with cow dung as additive) were synthesized in the Department of Chemistry, CBSH, Pantnagar, India. However, the exact constituting concentration of blend is not disclosed (under patent process). The compounds (in form of powder and films of size 1 × 1 cm^2) were investigated as the primary carbon source for tested bacteria. Nutrient broth (Hi Media, Mumbai, India) was taken as medium for the bacterial growth. For *in vitro* studies, Minimal Broth Davis without dextrose (Minimal Broth) (containing grams per litre: 7.0 K_2HPO_4; 2.0 KH_2PO_4; 0.5 $Na_3C_6H_5O_7$; 0.1 $MgSO_4.7H_2O$ and 1.0 $(NH_4)_2SO_4$) (Hi Media, Mumbai, India) medium was used which is deprived of carbon source.

Bacterial consortium

The selected bacterial cultures were retrieved from the departmental culture collection of Microbiology, CBSH, G. B. Pant University of Agriculture and Technology, Pantnagar, India (Table 2). These were originally isolated from different artificial soil beds and further characterized by 16S rDNA sequencing and the sequences were submitted to GenBank, NCBI. These cultures were selected based on their pre-identified potential to degrade a variety of polymers like LDPE (Kapri et al., 2010), HDPE (Satlewal et al., 2008), Epoxy, and Epoxy silicone blends (Negi et al., 2009) and PVC (Anwar et al, 2013). These bacterial strains *viz*. *Bacterium*Te68R strain PN12 (DQ423487), *Microbacterium* sp. strain MK3 (DQ318884) and *Pseudomonas putida* strain MK4 (DQ318885) were characterized and maintained as glycerol stocks. Further, an aliquot of 200 µL was withdrawn from glycerol stocks and the cultures were revived by inoculating into 10 mL of nutrient

broth (HiMedia, India) test tube at their optimum conditions and maintained on nutrient agar (HiMedia, India) at optimum pH (7.0 ± 0.2) and growth temperatures. Based on the preliminary nutritional screening, the bacterial consortium was developed. For this purpose, a single colony from each culture was inoculated into 20 mL flask containing 10 mL of nutrient broth (pH 7.0 ± 0.2) and the flasks were incubated at 37°C for 16 h with continuous shaking at 120 rpm to prepare active cultures which should be in mid-log phase of their growth. Further, the equal calculated amount of colony forming unit per mL (CFU/mL) of each strain was mixed for the development of consortium as described by Goel et al., 2011. The compatibility of the each used strains was also tested for the preparation of consortium and reported earlier by our research group (Sah et al., 2011).

Determination of optimum tolerance level of Epoxy and CME

The optimum concentration of Epoxy and CME, tolerated by the consortium was determined. For this purpose, the bacteria inoculated in the Nutrient Broth to prepare active culture, as mentioned previously in section of bacterial consortium. The active culture (an aliquot of 20 µL, OD=0.40) was inoculated into 96-well cell culture plate containing 200 µL Minimal Broth Davis without Dextrose per well. The crushed Epoxy and CME were then added at increasing concentrations from 0 to 8 mg/mL. Cell culture plate was incubated at optimum growth temperature (37±1°C) with continuous shaking (120 rpm) for overnight. The optimum tolerance level was further confirmed by inoculation of respective bacteria with 5 mL minimal broth into test tubes. And the similar concentrations of tested compounds were used in each tube and then incubated at optimum growth temperature (37±1°C) with continuous shaking (120 rpm) for overnight. The absorbance was recorded for all the treatments at 600 nm wavelength with ELISA plate reader and spectrophotometer after filtration (using whatman filter paper of 2.5 µm particle retention size) of non-biodegraded compound for 96-well plate and test tubes, respectively.

***In vitro* biodegradation studies of Epoxy and CME using consortium**

For this purpose, crushed Epoxy and CME samples were surface-sterilized with 70% ethanol for 10 min and subsequently dried under vacuum. The dried samples were added to 200 mL autoclaved minimal broth in 500 mL Erlenmeyer flasks. A total volume of 300 µL of active culture was then added to the minimal broth. Minimal broth with Epoxy and CME (5 mg/mL of medium i.e. 1.0 gm) were served as the negative control for their respective treatments. The flask having bacterial culture served as the positive control. And the flask containing Minimal Broth Davis with culture and crushed Epoxy/CME (5 mg/mL of medium) served as treatment. Flasks were incubated at optimum growth temperature (37±1°C) with continuous shaking at 120 rpm.

Statistical analysis

The *in vitro* experiments parameters viz. OD600, CFU/mL and λ max were performed with three replicates per treatment. Data were analyzed by ANOVA. Mean difference of the treatments was considered to be significant at the 5 % level.

Recovery of compounds after biodegradation and analysis

The treated polymer samples were recovered from the broth after four days of incubation. Then, the broth was filtered (using whatman filter paper of 2.5 µm particle retention size) and the bulk surface sample (residue) was recovered from filtrate. Filtrate was centrifuged at 8000 rpm (Sigma) for 10 min at 4°C. Pellet was discarded and supernatant was kept in oven at 60°C for overnight to evaporate the water from the dissolved compound. After evaporation, the dried biodegraded sample along with the remaining residue was used for spectral and thermal analysis through Fourier transform infrared spectroscopy (FT-IR) and simultaneous thermogravimetric-differential thermogravimetry-differential thermal analysis (TG–DTG–DTA), respectively.

Fourier transform infrared (FT-IR) analysis

Treated epoxy and CME compounds were analyzed by FT-IR (Perkin Elmer spectrum version 10.03.06) and different peaks relative to CH_2 deformation, CH_2 bending (symmetrical), CH_2 bending (asymmetrical), CH_2 stretching, CH stretching and C-O bond formation were compared taking untreated Epoxy and CME as the reference.

Simultaneous differential thermal analysis (TG–DTG–DTA)

TG-DTG-DTA analysis was done on a TG analyzer (EXSTAR TG/DTA 6300) from 30 to 800°C under a nitrogen atmosphere (200 mL/min) using a heating ramp of 10^0C/min in an Alumina pan.

***In situ* biodegradation studies**

Preparation of bioformulation

The rationale of using bioformulation was to maintain bacterial cell viability under adverse environmental conditions (sunlight-UV exposure, temperature fluctuation due to seasonal changes and inhibitory action of indigenous population etc.), so that they can persist longer in the soil under the *in situ* experiment. In addition, it also facilitates the easy transport; direct application; proper mixing in the soil and accessibility anytime at the site of action. For this purpose, Talc (composed of talcum steatite, talc fine powder and hydrous magnesium silicate) was purchased from Himedia Lab Pvt Ltd, Mumbai, India. The active consortium (600 mL) was prepared as discussed in section of bacterial consortium and the culture was then centrifuged at 15000 rpm for 10 min to remove the bacterial cells. Later, the supernatant was decanted and the pellets were added to 30 g of talc under sterile conditions and mixed properly. The mixture was then emptied into glass petri-plates, and kept at room temperature (28±1°C) aseptically for air-drying. For storage the bioformulation were kept at cool and dry place and subsequently checked for viability.

Shelf life of bioformulation

The viability of bacterial isolates in the formulation was ascertained by serial dilution plating method. For this purpose, one gm of talc-based formulation was dissolved in 10 mL of sterile distilled water and then subsequent dilutions were prepared up to 10^{-7} dilution. The dilution plating was done in nutrient agar medium. The plates were incubated at 37±1°C and the viability was checked initially after 2 and 4 days and then after regular interval of 7 days for subsequent 3 weeks followed by 15-days interval up to 180th day of storage of bioformulation as performed earlier by Sah et al., 2011. The above pattern was followed keeping in view the rapidity of

Table Sd1. Optimum tolerance level of polymer observed against used bacteria in the study.

Bacterial strain		*CFU/mL at the increasing concentration of Epoxy and CME (mg/mL)								
		0 mg/mL	1 mg/mL	2 mg/mL	3 mg/mL	4 mg/mL	5 mg/mL	6 mg/mL	7 mg/mL	8 mg/mL
MK3	Epoxy	36×10^{1}	33×10^{2}	30×10^{3}	43×10^{4}	33×10^{6}	64×10^{7}	50×10^{5}	33×10^{4}	66×10^{3}
	CME	50	33×10^{1}	38×10^{2}	70×10^{3}	31×10^{4}	50×10^{6}	64×10^{4}	30×10^{3}	60×10^{2}
MK4	Epoxy	39×10^{1}	44×10^{2}	34×10^{3}	63×10^{4}	33×10^{6}	33×10^{7}	40×10^{5}	36×10^{4}	55×10^{3}
	CME	45	33×10^{1}	34×10^{2}	39×10^{3}	40×10^{4}	64×10^{6}	60×10^{4}	37×10^{3}	62×10^{2}
PN12	Epoxy	45×10^{1}	30×10^{2}	346×10^{3}	60×10^{4}	33×10^{6}	160×107	33×10^{5}	56×10^{4}	78×10^{3}
	CME	40	33×10^{1}	37×10^{2}	32×10^{3}	43×10^{4}	70×10^{6}	68×10^{4}	34×10^{3}	54×10^{2}

*The data are average of triplicate experimental values**.**

changes in viable counts during storage. The plate counts were carried out in triplicates and the CFU/mL was calculated as the average of three readings.

Biodegradation assay

For *in situ* biodegradation assay, the top soil was dug from a barren land at Pantnagar, India and filled into 45×34 cm^2 sinks. The Epoxy and CME film coupons of dimensions 1×1 cm^2 were surface sterilized with 70% ethanol for 10 min and dried in vacuum. The dried films were then placed at various depths below the soil surface in respective treatment. The prepared bioformulation were added to the soil and then incubated in natural condition. Further, autoclaved distilled water was sprinkled at regular intervals of two weeks to maintain the moisture content of the soil. Aeration conditions were maintained by shoveling the soil at regular intervals. Moreover, the untreated pure Epoxy and CME coupons were taken as control for both the soil incubated polymer samples either in presence and absence of consortium.

Recovery of compounds from soil bed and analysis

The treated polymer film samples were carefully recovered from the soil after a period of subsequent three and six months. The recovered films were washed with sterile water to remove soil and contaminant and then subsequently dried under vacuum. The dried biodegraded samples were used for spectral and morphological analysis through FT-IR and Scanning Electron Microscopy (SEM), respectively.

Fourier transform infrared (FT-IR) analysis

The Epoxy and CME film samples were analyzed by FT-IR as described previously for *in vitro* biodegradation studies.

Scanning electron microscopy (SEM) analysis

For this analysis, the film samples were metallized with gold particles and analyzed by SEM (JEOL JSM-6610 LV) at 15.00 kV EHT under two successive magnifications (1.00 and 3.00 KX).

RESULTS

Determination of optimum tolerance level of epoxy and CME

The preliminary screening of epoxy and CME for their toxicity against used bacteria was observed that the optimum tolerance level of these polymers for respective bacterial strains was found to be 5 mg/mL (Table Sd1). Thus, the given concentration of polymers was used for the further *in vitro* biodegradation studies.

Comparative growth profiling of consortium in presence and absence of epoxy and CME

The growth profile of consortium was compared in the presence and absence of epoxy and CME (Figure 1). It was shown that epoxy did not affect the growth of bacteria, as the stationary phase was achieved within 3 days itself and bacterial biomass as depicted by the OD_{600} (Figure 1a and Table Sd2) and CFU/mL (Figure 1b and Table Sd3) was comparatively higher in presence of Epoxy (5 mg/mL). However, it was found that CME interfered the growth pattern of the bacteria as the stationary phase was achieved earlier i.e. within 48 h of the incubation (Figure 1a) and CFU count was lower in comparison to the controls (Figure 1b). This effect may be due to the organic matter and antibacterial property of cow dung (Waziri and Suleiman, 2013). Moreover, the significant increase in the OD_{600} was observed in the presence of CME, as the additive of the blend i.e. cow dung tended to dissolve in water and imparted a yellowish color to the medium resulted to increase in OD_{600} (Figure 1a). Thus, the comparative account reflected that the bacterial growth was better in the presence of Epoxy, as compared to CME.

In vitro biodegradation assay of Epoxy and CME using consortium

In the absence of consortium, Epoxy has shown the constant λ max during the incubation period (Figure 2 and Table Sd4). However, in presence of consortium, a shift in λ max of Epoxy from 218 to 222 nm within 2 days of incubation period and further to 216 nm within 4 days has illustrated the changes in the backbone of the

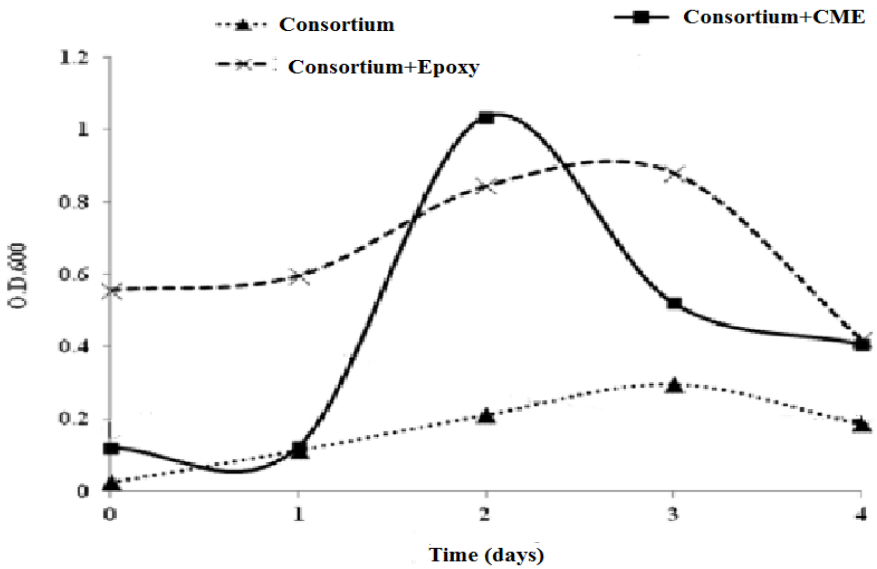

Figure 1a. Growth profiling of bacterial consortium in presence and absence of Epoxy and CME

Table Sd2. Two-way ANOVA of growth profiling of bacterial consortium in presence and absence of Epoxy and CME (using STPR2 software).

Day	Consortium OD600[a]	Epoxy+Consortium OD600[a]	CME+Consortium OD600[a]
0	0.023± 0.0011547	0.556 ±0.0008819	0.12±0.011547
1	0.112±0.001	0.595±0.0020817	0.123±0.0017321
2	0.21±0.0152753	0.843±0.002	1.035±0.0015275
3	0.296±0.0028868	0.88±0.011547	0.52±0.0173205
4	0.185±0.0008819	0.421±0.0034641	0.407±0.0017321

SEM: A (Treatment): 0.00322; B (Days): 0.00417; AXB (Interaction): 0.00722. CD (5%): A (Treatment): 0.00935; B (Days): 0.00120; AXB (Interaction): 0.00209. a, mean of three replicates. Data were analyzed statistically at the 5% ($p>0.05$) level of significance.

polymer. Moreover, CME has shown similar pattern of change in λ max either in presence (275 to 215 nm) or absence (280 to 220 nm) of the consortium during the incubation period of 4 days (Figure 2). It may be due to the organic content of CD, tends to dissolves in to the broth during the incubation period. Thus, these results demonstrated that the bacterial consortium has not imparted any significant change in the polymeric backbone of CME in the liquid medium. This may be due to the anonymous properties of cow dung which hampering the bacteria to act upon the CME.

Analysis of *in vitro* treated compounds

FT-IR spectra

FT-IR absorptions (cm^{-1}) of epoxy and CME are shown in Figure 3. Untreated Epoxy (Epoxy-U) has shown peaks corresponding to vOH (3435.58), $vasCH_3$ (3019.52), $vasCH_2$ (2966.43- 2400.38), vC=O (1720.87), δOH (1606.35), δNH (1509.77), $\delta asCH_2$ (1470.84), δCH (1384.52), γCH (1290.39, 1216.82), δC-C (1123.67), vC-O-C (1075.55, 1039.80), ρCH_2 (772.03) and vC-C-C (669.3, 626.85) (Figure 3a). The untreated CME (CME-U) has shown peaks corresponding to vOH (3435.97), δOH (1634.95), γCH (1219.47), ρCH_2 (772.21) and vC-C-C (685.31, 673.82) (Figure 3c). Such characteristic absorptions in the IR spectra of Epoxy and CME clearly indicated their synthesis. And the shift in the absorption frequency in CME to higher wave number clearly indicated the presence of CD in the Epoxy matrix. The consortium treated epoxy (Epoxy-T) has shown peaks corresponding to vOH (3798.52-3443.89), $vasCH_2$ (2342.75, 2143.06), δOH (1635.91), γCH (1218.91), vC-O-C (1074.06), ρCH_2 (771.96) and vC-C-C (685.27, 673.34) (Figure

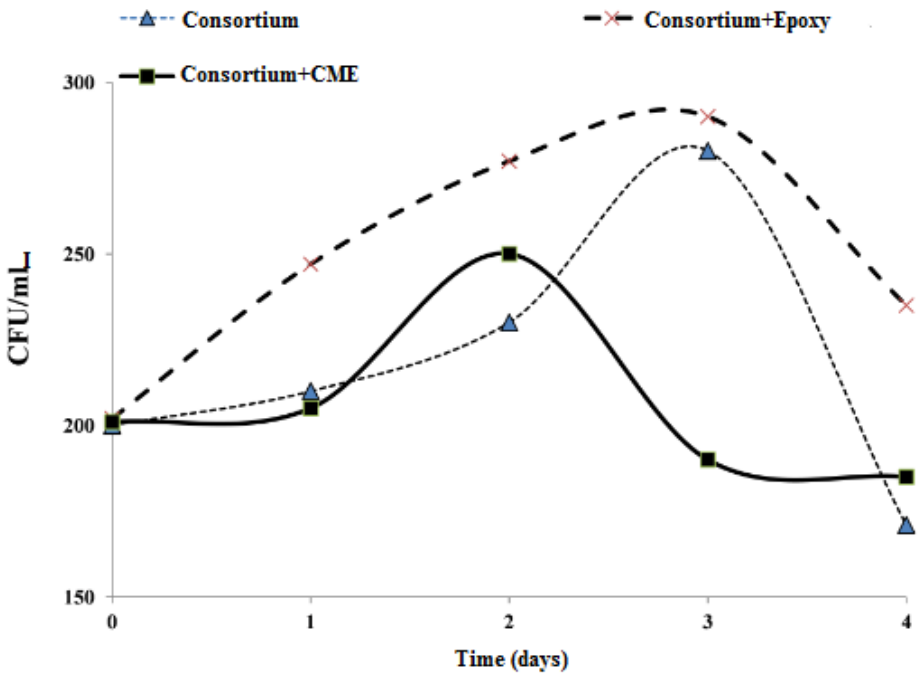

Figure 1b. Comparative analysis of colony forming units per ml (CFU/mL) in presence of Epoxy and CME with respect to control.

Table Sd3. Two-way ANOVA of comparative analysis of colony forming units per ml (CFU/mL) in presence of Epoxy and CME with respect to control (STPR2 software).

Days	Consortium CFU/mL[a]	Epoxy+Consortium CFU/mL[a]	CME+Consortium CFU/mL[a]
0	200±0.5773503	200±1.1547005	200±1.1547005
1	212±1.1547005	242±1.1547005	205±1.1547005
2	225±2.081666	275±2.8867513	250±2.8867513
3	270±1.1547005	285±1.4529663	170±0.5773503
4	175±0.8819171	230±1.7320508	185±2.081666

SEM: A (Treatment): 0.718; B (Days): 0.927; AXB (Interaction): 1.606. CD (5%): A (Treatment): 2.080; B (Days): 2.686; AXB (Interaction): 4.652. a: Mean of three replicates. Data were analyzed statistically at the 5% ($p>0.05$) level of significance.

3b). The disappearance of peaks at wave numbers 3019.52 (vas CH_3), 1720.87 (vC=O), 1509.77 (δNH), 1470.84 (δCH, asymt), 1384.52 (δCH) and 1123.67 (δC-C) with simultaneous increase in the wave number of vOH from 3435.58 to 3798.52 for Epoxy-T (Figure 3b) indicated its degradation.

The consortium treated CME (CME-T) has shown peaks corresponding to vOH (3435.82), vasCH_2 (2400.05, 2143.87), δOH (1637.38), γCH (1219.34), vC-O-C (1049.34), ρCH_2 (772.27) and vC-C-C (672.97) (Figure **3**d). The comparative study of the IR spectra revealed that the presence of consortium has enhanced the hygroscopic character of CME-T due to the appearance of absorption frequency corresponding to water molecule ranging 1634.95-1637.38 cm^{-1}. However, simultaneous increase in the wave number of vOH was not found for treated CME as compared to Epoxy-T, illustrating no structural change in CME during the incubation period of 4 days into the liquid medium. Further, all the samples were hygroscopic with banding vibration of H_2O from 1606.35 to 1639.28.

Simultaneous TG–DTG–DTA

The changes in the weight of treated compounds at

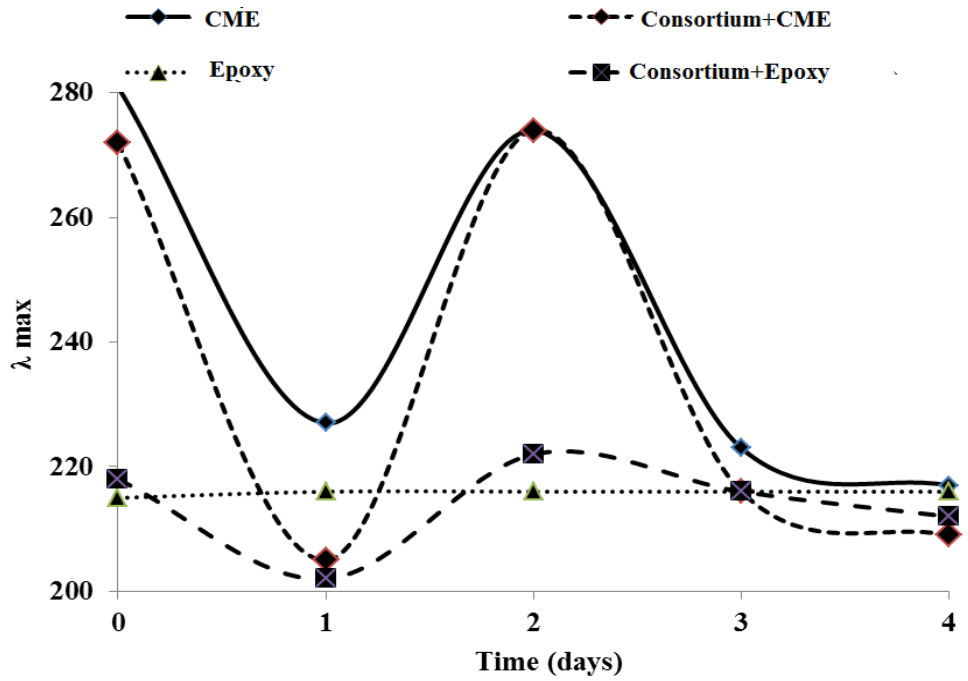

Figure 2. Comparative *in vitro* biodegradation assay of Epoxy and CME using bacterial consortium

Table Sd4. Two-way ANOVA of comparative *in vitro* biodegradation assay of Epoxy and CME using bacterial consortium (using STPR2 software).

Day	Epoxy λmax[a]	CME λmax[a]	Epoxy+Consortium λmax[a]	CME+Consortium λmax[a]
0	212± 1.1547005	281±0.5773503	221±0.8819171	272±1.1547005
1	212±1.7320508	227±1.1547005	202±1.1547005	205±1.7320508
2	213±2	274±1.5275252	225±1.1547005	274±0.5773503
3	213±2.081666	223±1.1547005	215±2.3094011	216±1.7320508
4	213±0.5773503	217±0.5773503	211±1.1547005	209±0.5773503

SEM: A (treatment): 0.595; B (days): 0.665; AXB (interaction): 1.33. CD (5%): A (Treatment): 1.704; B (Days): 1.905; AXB (interaction): 3.811. a: mean of three replicates. Data were analyzed statistically at the 5% (p>0.05) level of significance.

defined temperatures and time intervals and their thermal stability was observed through thermal gravimetric analysis. The simultaneous DTA-DTG-TG and thermal data of the consortia treated Epoxy and CME samples with reference to untreated Epoxy and CME as controls has been summarized in Figure 4 and Table 3, respectively. Epoxy-U has shown two-step decomposition reactions (Figure 4a). First step decomposition of Epoxy-U started at 200°C with 94.41% residue. This was supported with a DTG peak temperature of 230°C with rate of decomposition 0.27 mg/min. The Second step decomposition started at 300°C with 79.49% residue. This was supported with a broad DTG peak at 349°C with rate of decomposition with 0.67 mg/min. A combined DTA peak was appeared at 329°C with signal voltage of 19.7 µV that shows heat of decomposition of Epoxy-U (-) 391 mJ/mg. A weight loss of 0.03% at 100°C was found due to moisture content. A rapid weight loss was observed in the temperature range 300-400°C leaving weight residue 38.71%. This may be assigned to the oligomeric Epoxy content formed during the process of curing. The weight loss from 400°C onwards was very slow. The decomposition of Epoxy-U was terminated at 700°C leaving char residue 3.13%.

Relatively, the Epoxy-T has shown reduced thermal stability over cured Epoxy resin (Figure 4b). A rapid

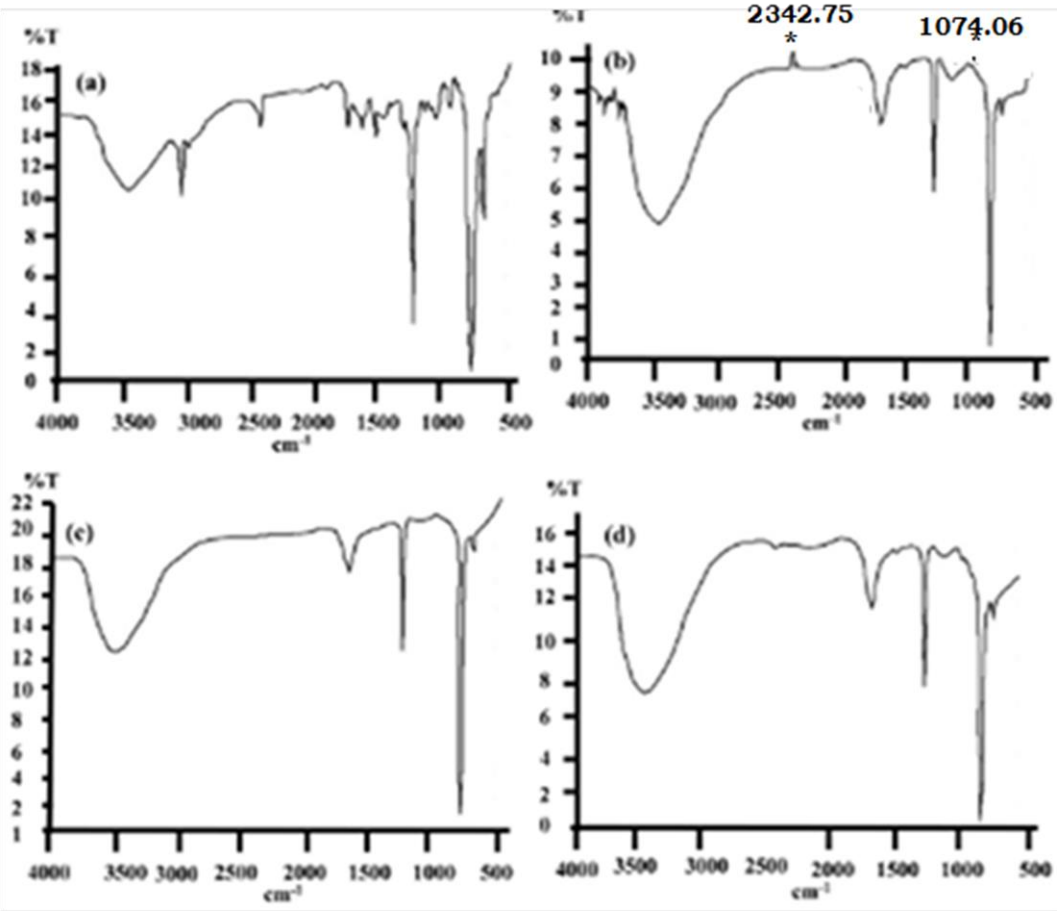

Figure 3. Effect of percentage transmittance on the wave number of (a) untreated and (b) treated Epoxy, (c) untreated and (d) treated CME, respectively during *in vitro* biodegradation studies.

weight loss was observed by Epoxy-T consisting of five-step degradation process. The rate of weight loss under defined conditions is inversely proportional to the size of the polymeric chains. And the 4.90% weight loss at 100°C was due to moisture content. The release of the moisture from the composite has been started at 62°C at the rate of 0.110 mg/min. The first step decomposition was appeared at 142°C leaving weight residue 90.9% with a sharp DTG peak at 151°C with rate of decomposition 0.274 mg/min. This has shown a DTA endotherm at 154°C with heat of fusion 56 mJ/mg. The second step decomposition was appeared at 200°C leaving weight residue 85.22% with heat of fusion 152 mJ/mg at 226°C. This was supported with a DTG at 213°C with rate of decomposition 0.141 mg/min. The decomposition of Epoxy-T further progressed leaving two minor profiles of degradation, which were appeared at 362°C with 0.149 mg/min and 637^{o}C with 0.59 mg/min. Further, the decomposition was ended with 57.20% char residue at 819°C.

The modification of Epoxy by cow dung has drastically reduced its thermal stability. But this has contributed a remarkable increase in the moisture content. The first step of decomposition of CME-U started at 200°C with 86.35% weight residue with a weak DTG peak temperature of 207°C with rate of decomposition 0.131 mg/min (Figure 4c). This was supported with a DTA at 214°C with heat of decomposition 334 mJ/mg at signal voltage (-) 14.8 µV. The second step decomposition reaction was started at 300°C leaving weight residue 79.33%. This was supported with a normal DTG at 320°C with rate of decomposition 0.164 mg/min. Due to hygroscopic contribution of cow dung to CME, moisture content of 8.77% was found. This was supported with a DTG at 72°C with elimination of water molecule at 0.243 mg/min rate of decomposition. The corresponding heat of dehydration of CME-U was appeared at 75°C with heat of decomposition 169 mJ/mg at signal voltage -15.2 µV. Weight loss at 300°C onwards was found to be very slow. The decomposition was terminated at 700°C leaving char

Table 3. Thermal analysis of treated and untreated Epoxy and CME.

Sample	DTG peak temperature		DTA	
			Endotherm	
	°C	Rate (mg/min)	°C	ΔH (mJ/mg)
Epoxy -U	230	0.27	329	-391
	349	0.67	-	-
Epoxy -T	62	0.110	154	56
	151	0.274	226	152
	213	0.141	-	-
	362	0.149	-	-
	637	0.59	-	-
CME-U	72	0.243	75	169
	207	0.131	214	334
	320	0.164	-	-
CME-T	77	0.284	79	201
	205	0.135	216	153
	332	0.174	-	-

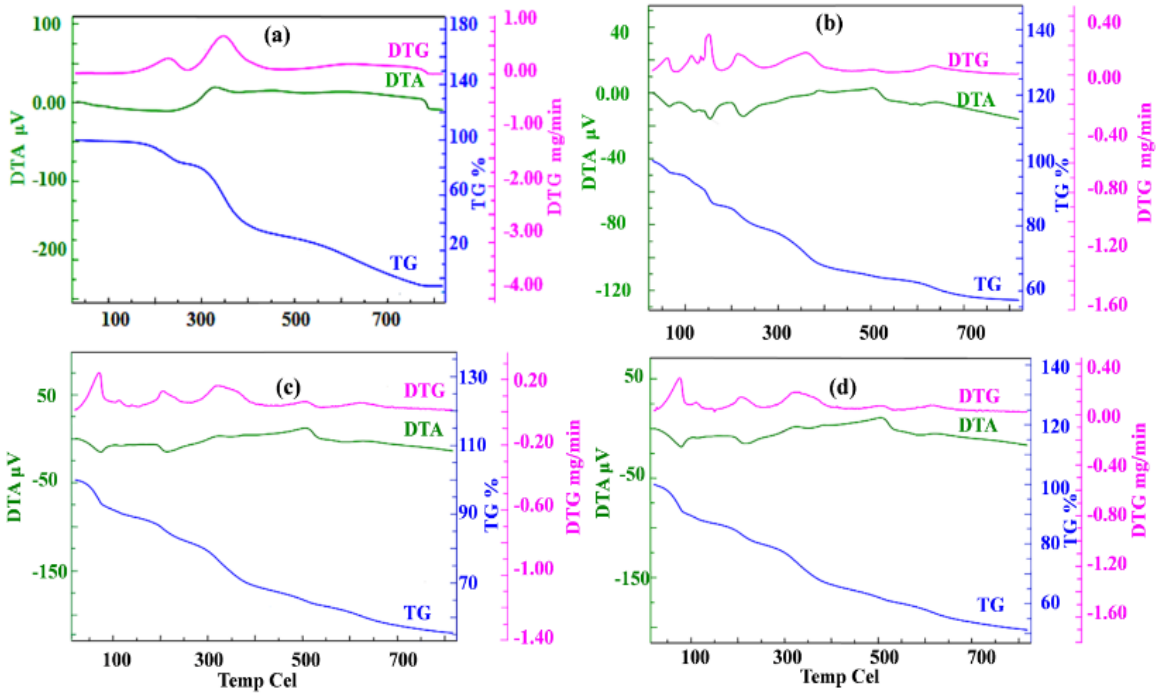

Figure 4. Thermal analysis of Epoxy-T (b) and CME-T (d) with reference to Epoxy-U (a) and CME-U (c), as their control, respectively.

residue 57.59%. The CME in the presence of consortium has shown similar degradation pattern as CME-U (Figure 4d). A comparative account of the thermogram of both the samples reveals that the presence of bacterial consortium has contributed to the reduced heat of fusion and greater moisture content over CME-U.

Shelf life of bioformulations

The viability of consortium was tested in talc-based formulation for a storage period up to 70 days. With the progression of the storage, consortium showed a sustained viability, whereby the counts dropped marginally from 279×10^6 to 253×10^6 after 70 days of storage. This consortium was found to be stable and viable as bioformulation. For commercialization, the viability of bioinoculants in a prescribed formulation for a certain period of storage is desirable (Bazilah et al., 2011). Similar studies of viability counts were also conducted on PGPR bioinoculants using sawdust as carrier (Arora et al., 2008). Talc based formulation has also been reported for PGPR strains for the storage and management of various plant pathogens (Shanmugam et al., 2011).

Analysis of *in situ* biodegraded Epoxy and CME

The comparative biodegradation of Epoxy and CME film was observed over 3 and 6 months of incubation using bioformulation in soil bed under natural conditions.

FT-IR spectra

FT-IR spectra of Epoxy (Figure 5) and CME (Figure 6) was conducted from 4000 to 500 cm^{-1}. Untreated pure Epoxy (Epoxy-P) has shown peaks corresponding to vOH (3435.58), vasCH_3 (3019.52), vasCH_2 (2966.43- 2400.38), vC=O (1720.87), δOH (1606.35), δNH (1509.77), δasCH_2 (1470.84), δCH (1384.52), γCH (1290.39), δC-C (1123.67), vC-O-C (1075.55), ρCH_2 (772.03) and vC-C-C (669.3, 626.85) (Figure 5a).

The epoxy treated in uninoculated soil (Epoxy-UN) for 3 months has shown peaks corresponding to vOH (3370.35), vasCH_2 (2920.40), δCH (1384.2), vC-O-C (1072.27) and ρCH2 (772.03) (Figure 5b). However, after 6 months of incubation the Epoxy-UN has shown peaks corresponding to vOH (3430.48), vasCH_2 (2958.89), vC=O (1733.26), δOH (1630.71), δasCH_2 (1455.29), δCH (1390.10), γCH (1250.01), δC-C (1164.13), vC-O-C (1066.52) and ρCH_2 (754.1) (Figure 5c). The spectral changes i.e. appearance of new peaks in Epoxy-UN spectra may have been occurred due to the inhabitant bacteria present in the soil and the prolonged incubation under soil bed. Environmental factors may also be assigned for these changes that is light, temperature, rain etc.

The epoxy treated in inoculated soil (Epoxy-T) for 3 months has shown peaks corresponding to vOH (3682.21), vas CH_3 (3019.02), vasCH_2 (2975.88, 2399.94), δOH (1601.53), δNH (1522.49), δasCH_2 (1476.19, 1422.55), γCH (1215.89), vC-O-C (1045.96), ρCH_2 (757.01) and vC-C-C (669.16, 627.14) (Figure 5d). However, after 6 months of incubation Epoxy-T has shown peaks corresponding to vOH (3694.48, 36.59.45) vasCH_3 (3019.97), δOH (1626.87), δasCH_2 (1402.81), γCH (1215.91), vC-O-C (1069.04), ρCH_2 (759.01) and vC-C-C (669.29) (Figure 5e).

The spectral changes had occurred in both the cases (Figure 5a, d and 5a, b). However, comparing 5 (a, d) to 5 (a, b) it was found that the later observed more changes. Conclusively, it was observed that spectral changes are due to the inhabitant bacteria present in the soil and the prolonged incubation under soil bed. Environmental factors may also be assigned for these changes. If, the consortium have had imparted the negative effect, it may be depicted more pronounced in case of 6 months result (Figure 5c, e) but it was not observed. The biodegradation has been shown clearly in Figure 5e as the loss of peaks at vasCH_2 (2958.89), vC=O (1733.26), δCH (1390.10) and δC-C (1164.13) as compared to peaks shown in Figure 5c even though the same consortium has worked for prolonged time period. So, we can conclude that bacterial consortium has not imparted any negative effect on the indigenous population.

The untreated pure CME (CME-P) has shown peaks corresponding to vOH (3435.97), δOH (1634.95), γCH (1219.47), ρCH_2 (772.21) and vC-C-C (685.31, 673.82) (Figure 6a). The CME kept in uninoculated soil (CME-UN) for three months, has shown peaks corresponding to vOH (3683.07, 3620.45, 3437.19), vas CH_3 (3019.43), vasCH_2 (2974.67, 2400.2), vC=O (1724.61), δOH (1602.05), δNH (1518.13), δasCH_2 (1475.82, 1422.49) and γCH (1215.83), vC-O-C (1045.90), ρCH_2 (758.07) and vC-C-C (669.13, 627.29) (Figure 6b). However, after 6 months, the CME-UN has shown peaks corresponding to vOH (3788.11, 3401.62), vas CH_3 (3019.94), δOH (1616.91), δCH (1385.05) and γCH (1216.16), vC-O-C (1068.86), ρCH_2 (770.97) and vC-C-C (669.23) (Figure 6c).

The 3 months bacteria treated CME (CME-T) sample has shown peaks corresponding to vOH (3436.44), vas CH_3 (3019.38, 2400.15), vC=O (1727.31), δCH (1384.3), γCH (1215.63), vC-O-C (1046.08), ρCH_2 (757.27) and vC-C-C (669.16) (Figure 6d). However, subsequently after 6 months, CME-T has shown peaks corresponding to vOH (3788.09, 3403.30), vCH_2 (2927.51), vC=O (1727.43), δOH (1627.13), δCH (1385.03), vC-O-C (1068.68) and ρCH_2 (770.45) (Figure 6e).

The peak corresponding to vOH has a different appearance in Figure 6 (a) (b) and (d)). In case of pure CME (a) it is at vOH (3435.97), untreated CME (b) has vOH (3683.07, 3620.45, 3437.19) and CME-T (d) has vOH (3436.44). It means that lower OH frequency represents the hygroscopic nature. By the treatment of microbial consortia, it has retained its hydroscopic nature rather in case of CME-UN (b), it has loosed the same.

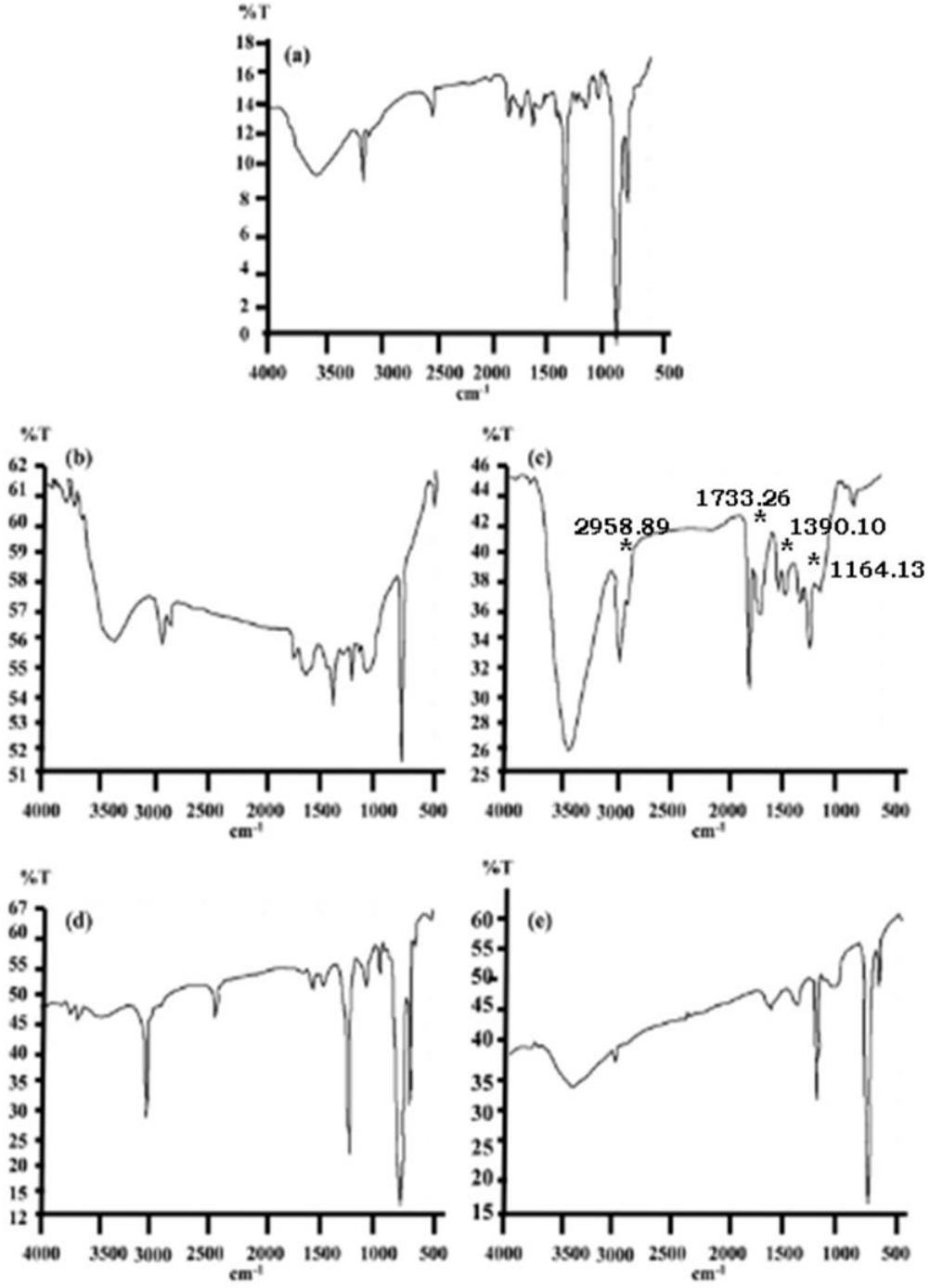

Figure 5. Effect of percentage transmittance on the wave number of pure Epoxy (a) Epoxy-UN (b), Epoxy-T (d) after 3 months and Epoxy-UN (c), Epoxy-T (e) after 6 months, respectively during *in situ* biodegradation studies.

There was not much significant change in the IR spectra of untreated and treated CME (Figure 6c and 6e) (500-1000). As only one peak corresponding to vC-C-C (669.23) has completely decomposed. Whereas, vas CH_3 (3019.94) and γCH (1216.16) has also vanished and vCH_2 (2927.51), vC=O (1727.43) are newly formed during treatment of microbial consortium. Conclusively, there was no significant change observed.

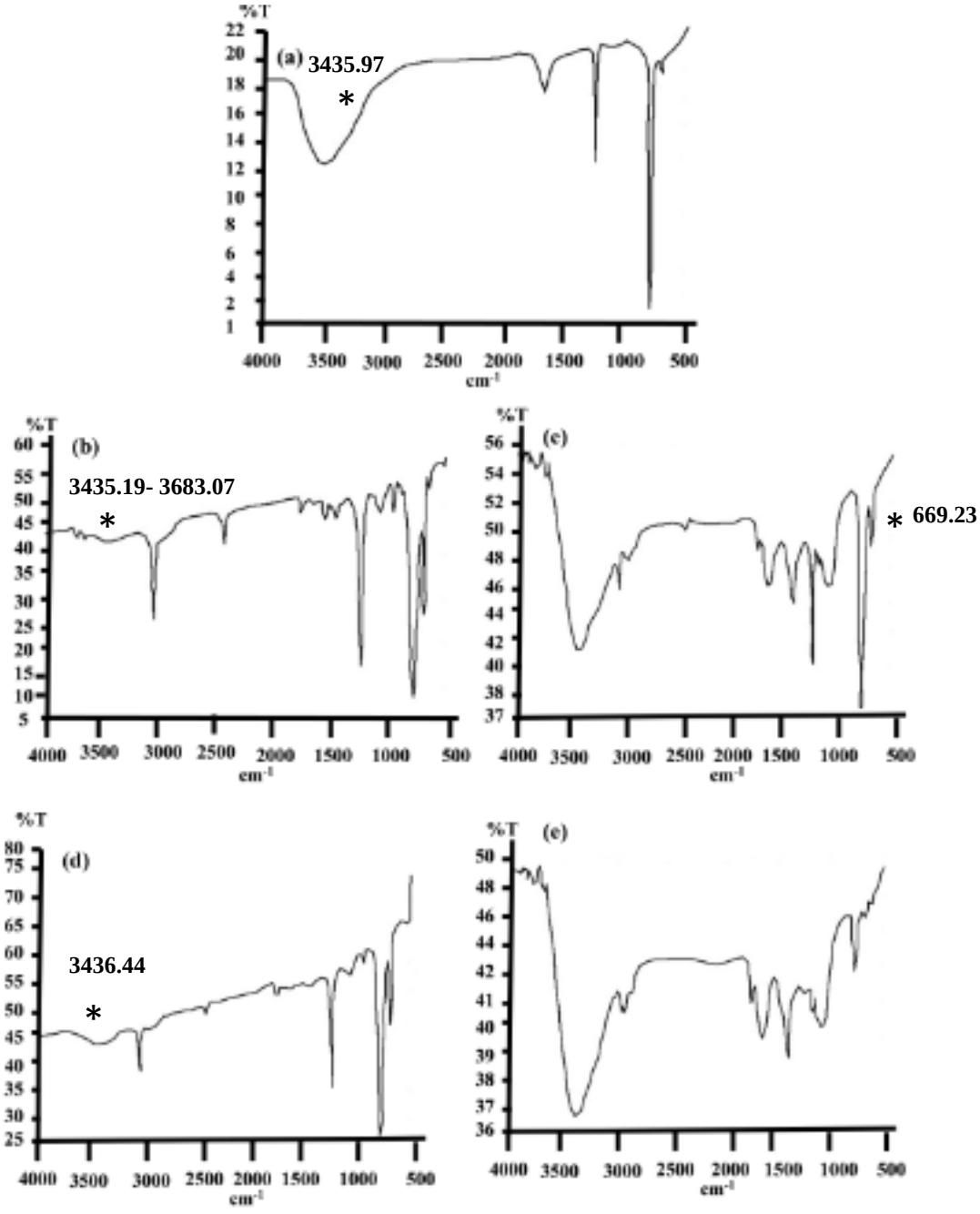

Figure 6. Effect of percentage transmittance on the wave number of pure CME (a) CME-UN (b), CME-T (d) after 3 months and CME-UN (c), CME-T (e) after 6 months, respectively during *in situ* biodegradation studies.

Scanning electron microscopy

The morphological changes on the surface of Epoxy (Figure 7) and CME films (Figure 8) due to the action of consortium were analyzed after incubation in soil for the period of 3 and subsequent 6 months. The untreated pure Epoxy film, which was taken as a reference, revealed smooth and homogenous morphology (Figure 7a). However, the Epoxy incubated in absence of consortium has been relatively changed after 3 months of incubation (Figure 7b). The abiotic factors like sunlight, rain, air and cooling under the soil bed may be responsible for the mechanical changes and heterogeneous surface of incubated film. Further, the occurrence of fissures, heterogeneous morphology and the surface resolutions and cracking of polymeric film were found to be

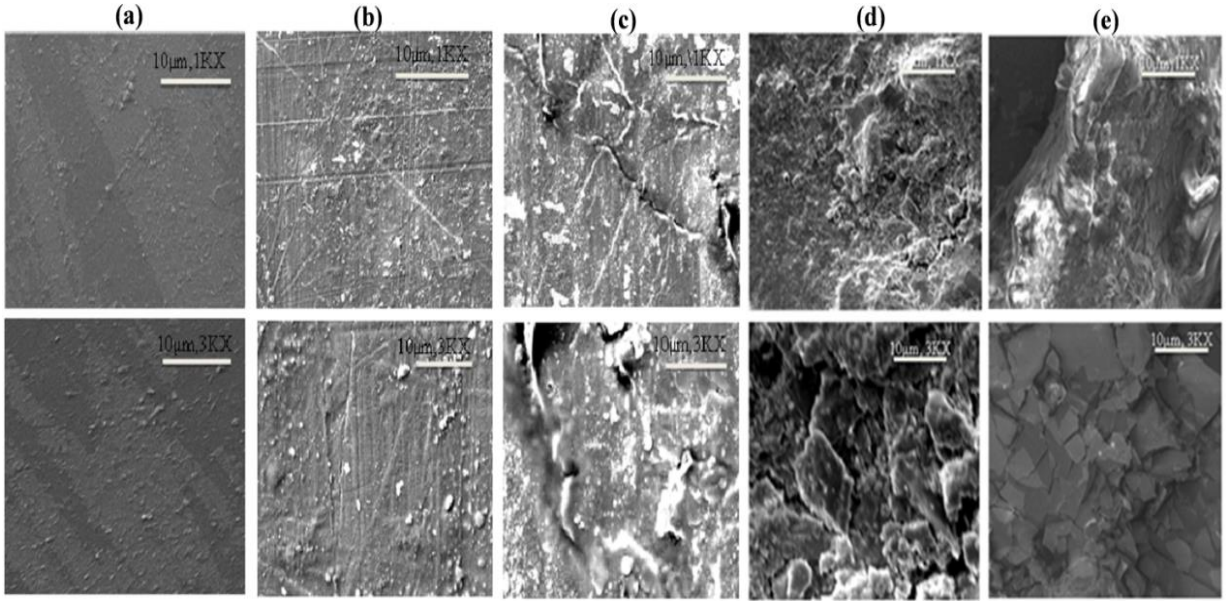

Figure 7. The morphology of the Epoxy film after incubation in soil; for 3 months in the absence (b) and presence (c) of consortia; for 6 months in the absence (d) and presence (e) of consortia, in reference to untreated pure Epoxy film as control (a). Scale bars=10 µm, 5 µm; Magnification=1.00, 3.00 KX, respectively.

remarkably increased in the epoxy film, incubated in consortium enriched soil (Figure 7c).

Subsequently, after 6 months of incubation, the well resolved worn-out areas with randomly distributed fissures may be either due to pressure of soil or the action of inhabitant microbes on the Epoxy film surface, incubated in absence of used bacteria (Figure 7d). Furthermore, in the bacteria enriched soil, the well resolved worn-out areas and cracks on the polymer surface were more pronounced (Figure 7e).

Similarly, the untreated CME film has also shown comparatively homogenous and smooth surface topography (Figure 8a). However, the changes in the CME films after incubation period of 3 months either without (Figure 8b) or with consortia (Figure 8c) observed as non-significant, may be due to the improved durability of CME film using cow dung as additive**.** Whereas, after 6 months of incubation period, the surfacial changes in CME film recovered from uninoculated soil, were observed (Figure 8d) and the surfacial changes were significant (as shown by well resolved fissures) in the film samples recovered from inoculated soil due to the action of used consortium on the polymer surface after the incubation period of 6 months (Figure 8e).

DISCUSSION

The present study was carried out with an aim of comparative *in vitro* and *in situ* biodegradation of Epoxy and Cow Dung Modified Epoxy (CME) using indigenously developed potential bacterial consortium. *In vitro* biodegradation assay revealed that the epoxy does not show any negative effect on the growth of bacterial strain during incubation period of 4 days in the liquid medium. In addition, it was observed that epoxy is degradable in the presence of used bacterial consortium which was apparent through changes in λ max, simultaneous shift in the wave numbers of various groups, reduced thermal stability, rapid weight loss and five steps degradation over untreated Epoxy resin as described by Negi et al (2009) as well.

However, the blend of Epoxy with cow dung that is CME interfered in the growth pattern and also rendered the growth of consortium within 2 days of incubation in liquid medium, as CFU/mL was found to reduce significantly. Further, no change in the λ max, structural and thermal property of CME, in the presence of bacterial consortium indicates that the used consortium was unable to cause any significant change in the polymeric backbone. It indicates the negative effect of used additive of blend that is cow dung on the bacterial growth (Waziri and Suleiman, 2013). Thus, the *in vitro* biodegradation studies revealed that the CME was not biodegraded by the used bacterial consortium during incubation period of 4 days. It may be due to the short period of incubation. In addition, the CME was used in crushed form and the interaction of bacteria to the compound was direct resulting to the

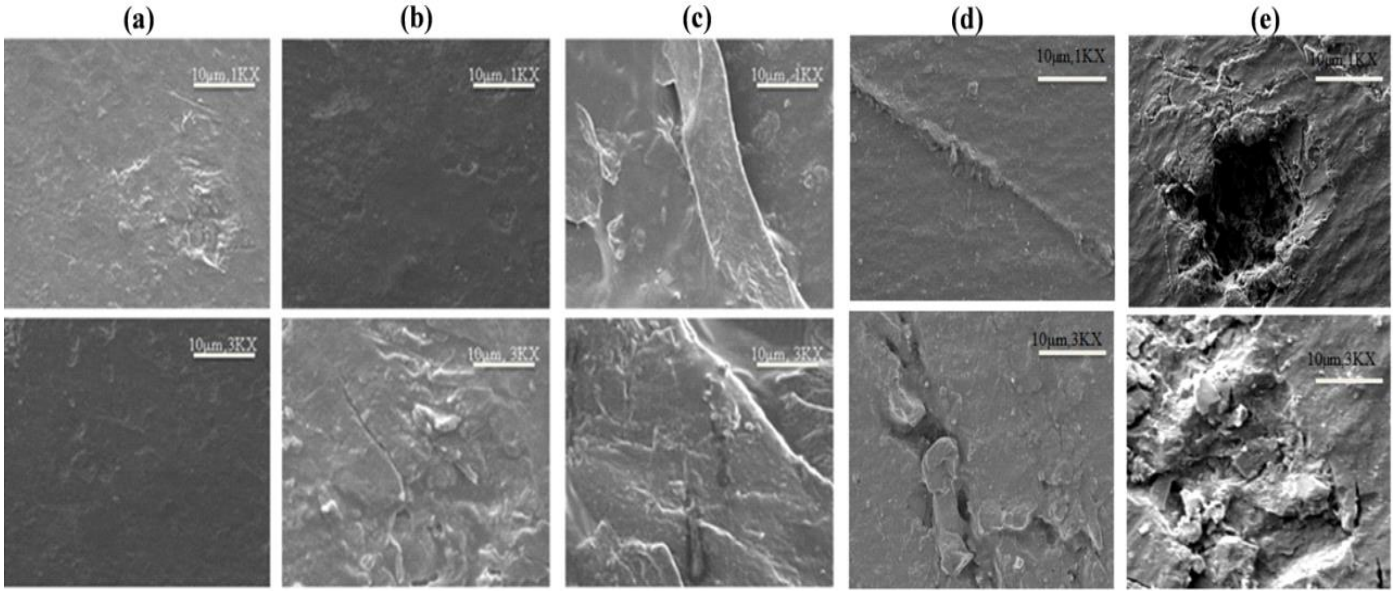

Figure 8. The morphology of the CME film after incubation in soil; for 3 months in the absence (b) and presence (c) of consortia; for 6 months in the absence (d) and presence (e) of consortia, in reference to untreated pure CME film as control (a). Scale bars= 10 µm, 5 µm; Magnification=1.00, 3.00 KX, respectively.

negative effect of cow dung on bacterial strains. However, the extent of biodegradation is also dependent on the physical form of the polymer. Thus, the *in situ* biodegradation study was conducted to observe the effect of natural conditions and natural microbial community with the indigenous bacterial consortium on both CME and epoxy films.

The Epoxy samples treated in natural condition for 3 months of incubation in presence of bacterial consortium showed occurrence of some new IR peaks and a remarkable decrease in vC-O-C with respect to its untreated control whereas, the spectral changes in the treated CME due to the consortium were non-significant upto 3 months of incubation. Thus, the comparative *in situ* biodegradation study indicated that the Epoxy surfacial degradation was achieved comparatively earlier and more pronounced in natural condition as the bacteria was able to act upon the epoxy polymeric backbone as depicted in the SEM micrographs. However, the additive cow dung interfere the bacteria to act upon the CME polymer backbone. Although, the morphological and spectral changes in CME film were achieved only after 6 months of incubation period.

Conclusively, the study found that the used bacterial consortium has the potential to utilize Epoxy under both *in vitro* and natural conditions as compared to its cow dung blend. This study also concludes that the CME samples could be degraded by potential bacterial consortium after keeping them in natural conditions for incubation period of more than five to six months.

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Conflict of interest

Authors hereby declare no conflict of interest.

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Optimization of conditions for generation of antimicrobial peptides from milk proteins by *Lactobacillus* spp.

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The batch growth conditions for the generation of antimicrobial peptides from skim milk were optimized by using Lactobacillus spp. Proteolysis being important factor for peptide production, 4 Lactobacillus strains were screened for proteolytic activity, two strains each from *Lactobacillus acidophilus* and *Lactobacillus delbrueckii*. *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09 with higher activity were selected to produce antimicrobial peptides. Incubation period (12-72 h), inoculum level (1-3%) and incubation temperature (37 and42°C) had an individual effect on antimicrobial activity of 10KDa Ultrafiltered skim milk hydrolysates checked against pathogenic *E. coli* ATCC 25922. Optimal conditions for generation of antimicrobial peptides include incubation period of 48 and 12-24 h, inoculums level of 2 and 1% and incubation temperature of 37°C for *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09 with proteolysis in range of 7.34-8.51 and 20-27.2 mMLeucine, respectively. Antimicrobial activity increased with proteolysis (>8.51 and >11 mMLeucine) but upto a certain extent after which it decreased for both strains. Maximum antimicrobial zone diameter obtained was 20 and 16.5 mm for *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09, respectively. The variables studied were very relevant due to their significance in improving the peptide production from both microorganisms and likely economic optimum conditions.

Key words: Antimicrobial peptides, fermentation, *Lactobacillus acidophilus, Lactobacillus delbrueckii*, optimization, proteolytic activity.

INTRODUCTION

The advanced development in the field of health and nutrition has directed focus towards 'food-derived bioactive peptides' that have regulatory functions in the human system beyond normal and adequate nutrition. Research in the field of bioactive peptides has intensified during the past two decades and it has been recognized that dietary proteins especially milk proteins are a rich source of biologically active peptides. Dziuba and Darewicz (2007) defined such peptides as inactive sequence of amino acids that are encrypted within the sequence of parent protein requiring proteolysis for their release from precursor. These are released during

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enzymatic digestion *in vitro* or *in vivo* (Pihlanto et al., 2010). Beneficial health effects of these peptides are due to their antimicrobial, antioxidative, antithrombotic, antihypertensive and immunomodulatory activities (Muller et al., 2008). All these biological peptides has its own physiological role but antimicrobial peptide has gained wide importance as emerging pathogens causing illness poses a threat to human health. Antimicrobial peptides are important component of innate immunity. Till date more than 880 such peptides are well recognised in international database. They can rapidly kill a broad range of microbes and have additional activities which impact on the quality and effectiveness of innate responses and inflammation (Rydengerd et al., 2008). Milk-derived antimicrobial peptides have high potential as supplements in functional foods or as food-grade biopreservatives or in medicinal use (Benkerroum, 2010).

Fermentation of milk using the proteolytic systems of lactic acid bacteria is an attractive approach for generation of bioactive peptides with low cost (Philanto, 2010). Such bioactive peptides are expected to be fundamentally different from those released by digestive proteinases which differ from microbial proteinases in specificity and mode of action (Benkerroum, 2010). Lactic acid bacteria (LAB) possess cell envelope-associated proteinases (CEP) and intracellular peptidases, which release bioactive health-beneficial peptides during food fermentation. Proteinases of lactic acid bacteria may hydrolyse more than 40% of the peptide bonds of bovine α_{S1} casein and β-caseins, producing oligopeptides of 4 to 40 amino acids residues (Kunji et al., 1996). The use of LAB for release of bioactive peptides encrypted in primary sequence of milk proteins is a promising strategy as it is already benefitted from “generally recognized as safe” status (Benkerroum, 2010). Among the *Lactobacillus* species, *Lactobacillus casei, Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus rhamanosus* and *Lactobacillus helveticus* are most extensively studied for release of bioactive peptides during fermentation.

Fermentation temperature significantly influences the bacterial growth, proteolytic activity and bioactivity. Otte et al., (2011) reported fermentation rate of *L. helveticus* MI1198 and *L. helveticus* CHCC 4080 increased with increasing temperature from 33°C to 40°C and the final pH decreased after 24 h suggesting that the optimum fermentation temperature for these strains may be higher than 40°C. Higher ace-inhibitory activity of *L. helveticus* strains at higher temperature point to the optimal growth temperature as the most efficient due to the largest number of bacterial cells and higher level of cell wall proteinase activity. The cell envelope proteinase, and aminopeptidase and X-prolyldipeptidyl aminopeptidase activity was maximum at 37- 40°C in another *L. helveticus* LB 10 strain (Pan and Guo, 2010). The good proteolytic activities with intra- and extracellular specific peptidases including X-prolyl-dipeptidyl aminopeptidase was also reported in different Lactobacillus strains that is *L. acidophilus*, *L. casei*, *L. rhmanosus*, *L. reutri*, *L. delbrueckii* and *Bifidobacterium* spp. at 37°C incubation temperature (Kholif et al., 2011). So in present study two fermentation temperatures [37°C (below 40°C) and 42°C (above 40°C)] were selected to check temperature effect on proteolytic activity which further can affect antimicrobial activity.

To date, only a few studies (Hayes et al., 2006; Tadesse et al., 2006; Lopez-Exposito et al., 2007) have reported antimicrobial peptides from fermented dairy products. However, no information is available on generation of antimicrobial peptides from skim milk by extended fermentation and varying growth conditions with Lactobacillus spp. Proteolytic system of LAB provide transport systems specific for amino acids, di- and tripeptides and oligopeptides of up to 18 amino acids. Longer oligopeptides which are not transported into the cells can be a source for the liberation of bioactive peptides in fermented milk products when further degraded by intracellular peptidases after cell lysis. Furthermore growth conditions affect the activity of proteolytic system of bacteria. Therefore by extending the fermentation period and varying the growth conditions peptide content as well as their activity can be enhanced. Hence, the present investigation was proposed with the view of exploring the proteolytic capability of lactobacilli to optimize the generation of antimicrobial peptides from skim milk.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Two strains of *L. acidophilus* NCDC 14, NCDC 15 and *L. delbreuckii* NCDC 08, 09 were procured from National Collection of Dairy Cultures (NCDC), National Dairy Research Institute (NDRI), Karnal and revived by propagating twice in de Man-Rogosa-Sharpe (MRS) broth at 37°C. One set of cultures was stored at -80°C in MRS broth containing 20% glycerol. Revived cultures were used to inoculate fresh MRS broth for experiment.

Growth media

For optimum cell envelope proteinase activity three different test media MRS, Minimal defined media (MDM) and bovine Skim milk were screened. On the basis of nutritional requirements of respective strains (Morishita et al., 1981) the minimal defined media was prepared from concentrated individual stock solutions of constituents stored at 4°C after filtration, except for the cysteine solution, which was freshly prepared. Stock solutions were composed of 100-fold-concentrated solutions of each amino acid, base, and vitamins. All amino acids, vitamins, purines, pyrimidines, and inorganic salts were of analytical grade (Sigma Chemical Co., S.D Fine, Himedia., Fischer scientific). Media and stock solutions were sterilized by filtration through a Durapore PVDF membrane (0.22-µm-pore size; Millipore Ireland Ltd.).

Bovine milk was collected from dairy farm, NDRI and fat fraction was removed in skim milk separator. 25 ml of skim milk was added in test tubes (50 ml volume) and sterilized in autoclave at 121°C

for 15 min and stored at 37°C for 24 h for sterility test.

Working culture was propagated in MRS broth at 37°C for 16 h. To eliminate carryover nutrients, the cells were harvested by centrifugation at 8,000 *g* for 15 min, washed twice in sterile 50 mM sodium phosphate (pH 7.0) and resuspended in buffer to the original volume. This cell suspension was used to inoculate the different media at an initial optical density OD_{580} of 0.07 whereas in skim milk inoculation it was on percent basis and incubated for overnight. Bacterial growth was monitored by measuring the OD_{580}. In the case of skim milk growth was monitored by diluting incubated SM media by a factor of 3.5 with 35 mM ethylene diaminetetraacetic acid adjusted to pH 12 (Kanasaki et al., 1975).

Cells grown in the MRS and MDM were harvested by centrifugation (10,000 g/10 min/4°C) at the exponential growth phase (OD_{580} 0.67) and washed twice with 0.85% (w/v) saline supplemented with 10 mM $CaCl_2$, and resuspended to final OD_{580} of approximately 10 in 100 mM sodium phosphate (pH 7.0). To harvest cells from skim milk 5 ml of 20% trisodium citrate/100 ml of inoculated skim milk was added, pH was adjusted to 7.0 with 10M NaOH and centrifuged (6000 rpm, 15 min, 4°C). Cell pellets thus obtained was washed twice with 50 mMTris –HCl containing 1% trisodium citrate (pH 7.0) and further processed as described above.

Enzyme assay

The proteinase activity of whole-cell suspensions resuspended in 100 mM sodium phosphate buffer (pH 7.0) at 37°C was measured with the chromogenic substrate succinyl-alanyl-alanyl-prolyl-phenylalanine-p-nitroaniline (Sigma) as described by Hebert et al. (2008) with slight modifications. The assay mixture, containing 200 µl of 50 mM sodium phosphate buffer (pH 7.0), 100 µl of 5 M NaCl (final concentration, 1.5 M), 20 µl of 20 mM substrate, and 60µl of the cell suspension, was incubated at 37°C for 10 min. The reaction was stopped by heating at 90°C, and mixture was centrifuged at 10,000 *g* for 5 min. The released nitroaniline was measured at 410 nm by using a microplate reader (Infinite F200 PRO). One unit of proteinase was defined as the amount required to liberate 1 nmol of nitroaniline per minute. Specific activity was expressed as units of proteinase per mg protein. Milk itself contains principal protease plasmin with other including cathepsins and elastase which can hydrolyse milk proteins (Kelly et al., 2006). So to get the net proteolysis carried out by harvested bacterial cells from milk, MRS and MDM endogenous activity of unfermented control milk as well as of MRS and MDM control was deducted.

Effect of process variables on proteolysis and antimicrobial activity by selected *Lactobacillus* spp.

Inoculated Skim milk media was used further to inoculate skim milk. Different process variables incubation time, inoculation level and incubation temperature were studied to investigate their effect on antimicrobial activity of hydrolysates.

A volume of 25 ml of sterilised bovine skim milk was inoculated on 2% basis with 12 h inoculated skim milk (exponential phase culture) and incubated at incubation temperature of 37°C. Samples were removed periodically at an interval of 12 h up to 72 h. Similarly different innocula sizes of 1, 2 and 3% (v/v) were studied only at 37°C up to 24 h. To check the effect of temperature, skim milk inoculated at 2% level was incubated at two different temperatures (37 and 42°C) for 24 h. Samples were stored at -20°C till further study.

Proteolytic activity

Proteolytic activity of fermented milk samples was measured using Ortho-pthaldehyde (OPA) method (Church et al., 1983). To 2.5 mL fermented milk, 0.5 mL distilled water and 0.5 mL of 0.72 $mol.L^{-1}$ trichloroacetic acid (TCA) were added and solution was filtered after 10 min. To 3 mL OPA reagent, 150 µL of sample (TCA filtrate) was added and incubated for 2 min at room temperature; the absorbance was measured at 340 nm using dual beam spectrophotometer (Specord 200, Analytik Jena AG, Germany). A calibration curve of leucine (0.25 - 2.5 $mmol.L^{-1}$) was prepared and the results were expressed as mmol L^{-1} leucine. One control sample without inoculation was also included in each experiment. For net proteolytic activity of inoculated sample, control sample's activity was deducted. *L. helveticus* NCDC 288 was taken as positive control as many previous studies revealed higher proteolytic activity of *L. helveticus* (Gandhi and Shah, 2014; Ramesh, 2011).

Antimicrobial activity

pH of the fermented milk sample was adjusted to 3.8 with 50% lactic acid and centrifuged at 10000 *g* /15 min at 4°C. pH of supernatant obtained was adjusted to 7 by 10 M NaOH, centrifuged and ultrafiltered through 10 KDa membrane (vivaspin). Hydrolysate thus obtained was used to check antimicrobial activity.

A well diffusion assay was performed using the indicator strain *E. coli* ATCC 25922 to screen antimicrobial activity of hydrolysates. The sensitivity of a strain was scored according to the diameter of the zone. The assay was carried out in the nutrient agar media (2% w/v) and 5 ml of soft agar media containing 10^4 CFU of an overnight culture of the indicator strain. Wells, 17 mm in diameter, were cut into solidified agar plates and 10 µl of soft agar was added to seal wells. Then 100 µl of the sterile 0.22 µm filtered samples were placed into each well. Plates were stored at 4°C for 1 h to permit radial diffusion of hydrolysates, incubated at 37°C for overnight, and subsequently examined for zones of inhibition. One control sample without inoculation was also included in each experiment. The net antimicrobial activity was obtained by subtracting inhibition zone of control milk from inoculated sample.

The effect of different fermentation conditions on bioactive peptide production from skim milk fermented by *L. acidophilus* NCDC 14 and *L. delbreuckii* NCDC 09 was studied and results are given below. The results were statistically analyzed by two way ANOVA. All three process parameters had a significant effect on bioactivity and peptide production. The study undertaken for the production of antimicrobial peptides by exploiting the proteolytic activity of Lactobacilli represents a novel and promising strategy and to best of our knowledge no study have been undertaken before.

RESULTS

Influence of growth media on CEP activity

The proteinase activity of *Lactobacillus* grown in MRS, MDM and Skim milk were compared. Among three media higher activity was observed in case of skim milk for *L. acidophilus* NCDC 14 (14.2 U/mg protein) followed by *L. delbrueckii* NCDC 09 (13.42 U/mg protein) being twenty fold higher than synthetic media MDM. However, the extent of increase in proteolytic activity in skim milk was different for each strain. In case of MRS broth proteinase activity was almost negligible as compared to MDM (Figure 1). On the higher proteinase activity basis, skim milk was selected as media to grow cells for antimicrobial peptide production from skim milk. *L. acidophilus* NCDC

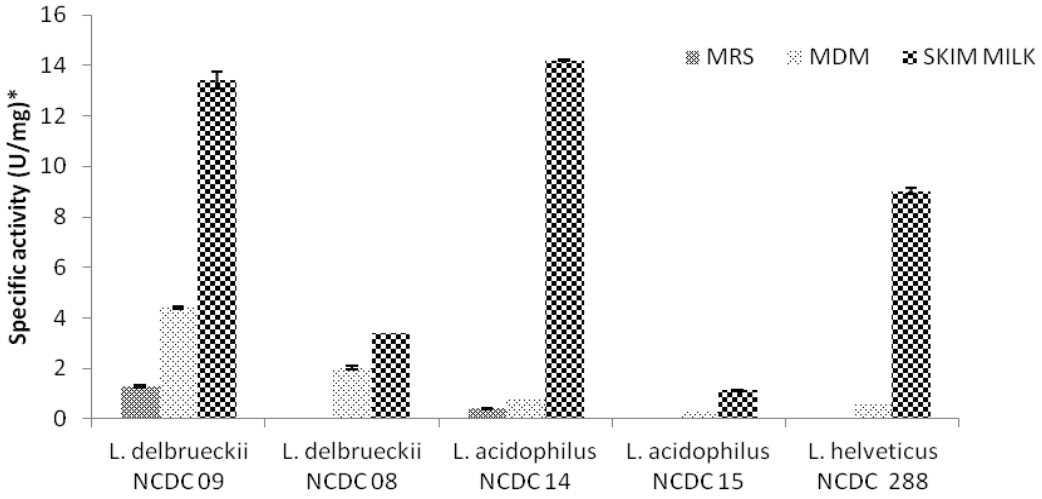

Figure 1. Effect of growth media on cell envelope proteinase specific activity of *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09. *One unit of enzyme (UE) was defined as the amount required to liberate 1 µmol of nitroaniline per minute. Specific activity was expressed as units of proteinase per mg protein.values are the mean±standard deviation (error bars) of three independent experiments.

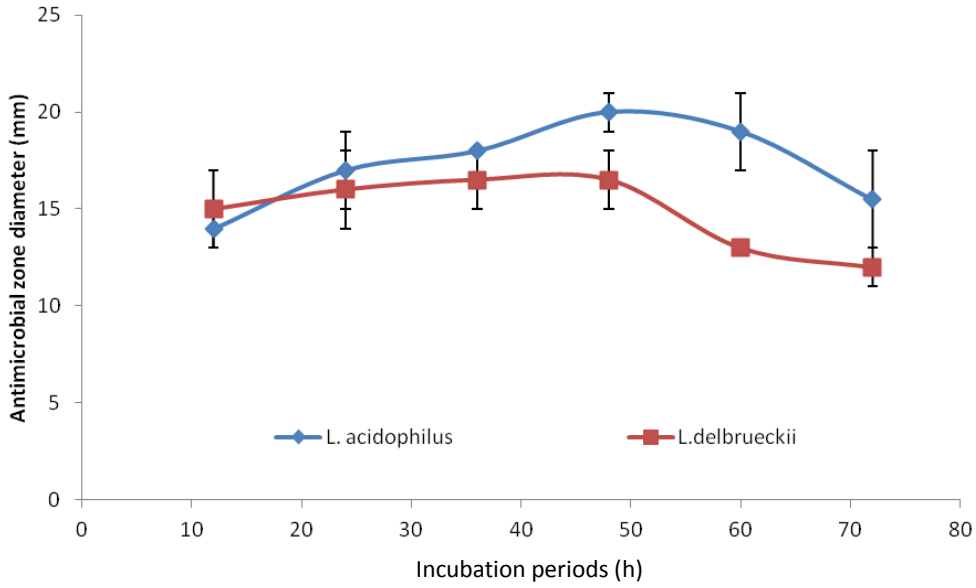

Figure 2. Antimicrobial activity (zone diameter) of skim milk hydrolysates obtained by fermentation at different incubation periods with *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09 against *E. Coli* ATCC 25922. Error bars show standard deviation.

14 and *L. delbrueckii* NCDC 09 showed higher proteinase activity in skim milk as compared to positive control *L. helveticus* NCDC 288 (9.02 U/mg protein).

Effect of incubation period on antimicrobial and proteolytic activity

For production of antimicrobial peptides three important fermentation variables i.e., incubation period, inoculation level and incubation temperature were optimized by three independent experiments by keeping other two variables constant.

In the current study, Figure 2, depicts the antimicrobial activity of skim milk fermented by *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09 at various time intervals (0, 12, 24, 36, 48, 60 or 72 h) during incubation at 37°C. The measured net inhibitory activities varied from zone

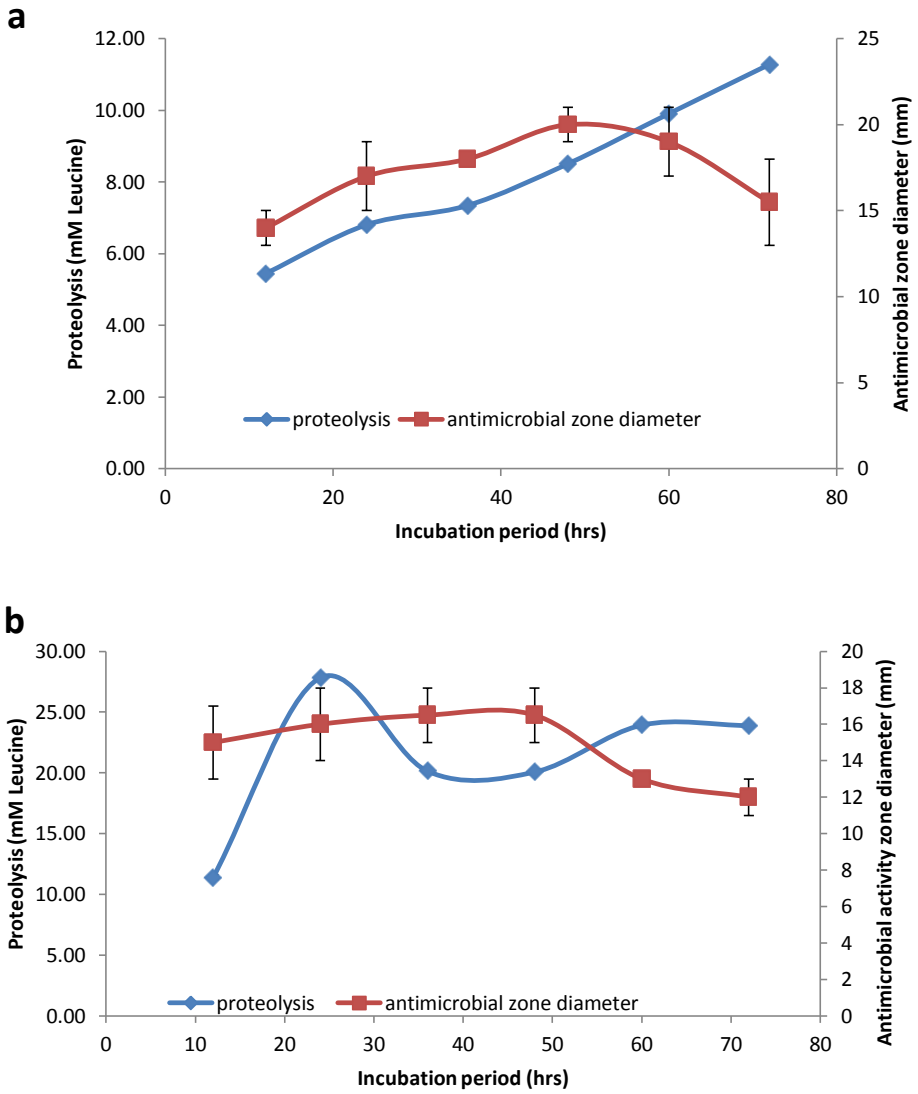

Figure 3. Effect of proteolysis on antimicrobial activity at different incubation periods of fermentation by *L. acidophilus* NCDC 14 (a) and *L. delbrueckii* NCDC 09 (b). Error bars show standard deviation.

diameter of 13 to 20 mm after deducting endogenous antibacterial activity of control milk during the fermentation time. There was no significant difference between the strains and reached a similar or relatively higher level of antimicrobial activity. The fluctuations were observed in antimicrobial activity at different incubation periods. In *L. acidophilus* NCDC 14 fermented milk antimicrobial zone diameter (14 mm) at 12 h of fermentation period increased to 20 mm at 48 h after which it decreased significantly ($P<0.05$) at the end of fermentation that is 60-72 h. Although, for *L. delbrueckii* NCDC 09 no significant difference was observed up to 48 h after which it decreased significantly.

The antimicrobial activity pattern observed for both strain could be associated with the proteolytic activity of strains. As depicted in Figure 3a and b the amount of liberated amino groups and peptides increased significantly ($P<0.05$) during fermentation from 12 to 72h for *L. acidophilus* while for *L. delbrueckii* upto only 24 h with higher degree of proteolysis within 12 h. Further in 24 h proteolysis increased at a higher rate and decreased thereafter up to 48 h with small increase again at the end of fermentation period. However for *L. acidophilus* NCDC 14 continuous and steady increase in proteolysis was observed. Peptide content for *L. acidophilus* NCDC 14 fermented skim milk increased significantly up to 36 h while insignificant differences were observed up to 60 h and increased again significantly at the end of fermentation period which is directly related to proteolysis.With increasing proteolysis peptide content also increased and was higher in *L. delbrueckii* NCDC 09.

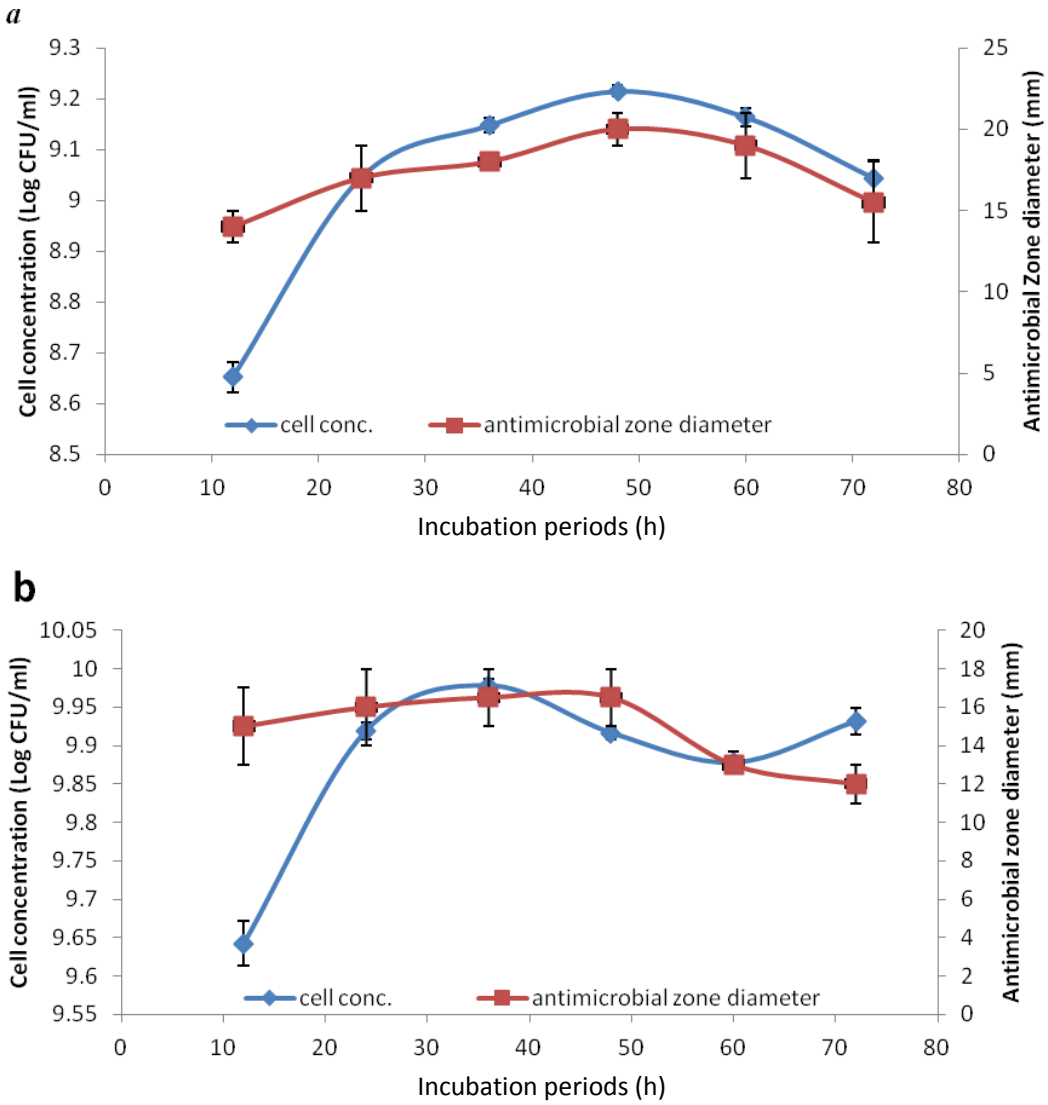

Figure 4. Effect of cell concentration on antimicrobial activity at different incubation periods of fermentation by *L. acidophilus* NCDC 14 (a) and *L. delbrueckii* NCDC 09 (b). Error bars show standard deviation.

Effect of incubation period on growth performance

The proteolysis further may be dependent on growth performance of the strains which may also affect the antimicrobial peptide production. Further positive correlation coefficient (r^2= 0.94, 0.77) among proteolysis and cell concentration for both strains also suggest this. Changes in growth and pH of all organisms at 12 h interval during growth in sterile skim milk at 37°C are shown in Figure 4a and b. The variation in initial count of different organisms is due to variation in growing ability of these organisms. There were greater differences in initial counts of *L. delbrueckii* NCDC 09 (7.73 log CFU) as compared to *L. acidophilus* NCDC 14 (6.4 log CFU). Both organisms showed increase in log counts (9.12-9.92) up to 36-48 h with corresponding decrease in pH (3.67). For *L. delbrueckii* NCDC 09 the drop of pH was much faster reaching 3.9 within 12 h of incubation with corresponding growth rate reaching to 10 log CFU/ml.

Cell concentration has great effect on proteolysis as well as antimicrobial activity. However at higher cell concentration antimicrobial activity may not be higher as in case of *L. delbrueckii* NCDC 09, as with higher proteolysis antimicrobial zone diameter decreased. At lower proteolysis, antimicrobial activity was higher with insignificant difference at higher proteolysis.

Effect of inoculum size on antimicrobial and proteolytic activity

In this study, inoculum size resulted in a significant effect

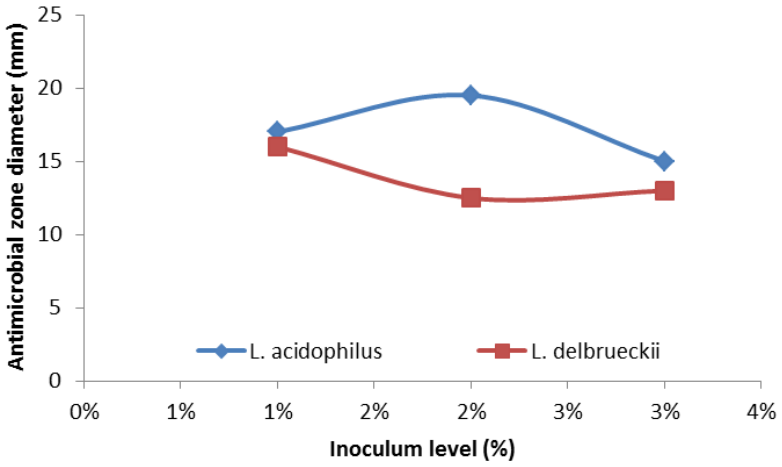

Figure 5. Antimicrobial activity (zone diameter) of skim milk hydrolysates obtained by fermentation at different inoculums levels with *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09 against *E. Coli* ATCC 25922.

on antimicrobial peptide production (Figure 5). At the given inoculum size range (1% to 3%, v/v), antimicrobial activity for *L. acidophilus* NCDC 14 increased with increasing size of the inoculum, from 1 (17 mm) to 2% (19.5 mm) and then decreased for 3% innocula size (15 mm) (Figure 6a). Instead, *L. delbrueckii* NCDC 09 (Figure 6b) showed higher zone diameter (16 mm) at 1% (v/v) and activity decreased further at 2-3% inoculum levels (12.5-13 mm). Based on these results, the optimal inoculum size for antimicrobial peptide production was determined to be 2 and 1% (v/v) for *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09, respectively of exponential phase culture, which gave the highest antimicrobial zone diameter (20 and 16 mm) against pathogenic *E. coli* ATCC 25922.

The inoculum size resulted in significant effect on proteolysis which varied among both strains also. The amount of liberated amino groups and peptides increased significantly ($p<0.05$) during fermentation at 1-3% inoculums levels for both strains. Higher degree of proteolysis and peptide content was observed at 3% level for *L. delbrueckii* NCDC 09 (23.9 mM Leucine and 6.23 mg/ml) followed by *L. acidophilus* NCDC 14 (4.21 mM Leucine and 2.43 mg/ml) with significant differences among 2 and 3% inoculums levels. With increasing proteolysis peptide content also increased and was higher in *L. delbrueckii* NCDC 09 (6.23 mg/ml) followed by *L. acidophilus* NCDC 14 (4.21 mg/ml).

Effect of Inoculum Size on growth performance

Both lactobacilli used at inoculation levels 1-3% successfully achieved the desired level and reached 9.07 and 9.12 log cfu/ml, respectively at the end of 24 h incubation period. *L. delbrueckii* NCDC 09 reached highest growth level with significant differences ($p< 0.05$) among inoculation levels 1%-3% (9.73, 9.96 and 10 log cfu/ml respectively) at the end of incubation period. *L. acidophilus* NCDC 14 also showed significant differences ($p< 0.05$) in cell concentration and decrease in pH at all inoculation levels. Highest pH decrease was observed for *L. delbrueckii* NCDC 09 (3.67) followed by *L. acidophilus* NCDC 14 (4.43). Comparison of pH values of fermented milks revealed that at 1 and 3% inoculation levels pH values were significantly different from each other being lowest at 3% for *L. delbrueckii* (data not shown). As shown in Figure 7a and b, higher cell concentration was observed at higher inoculums level for both *Lactobacillus* strains. Both strains at 3% (v/v) observed highest pH decrease and proteolytic activity but decreased antimicrobial activity.

Effect of Incubation temperature on antimicrobial and proteolytic activity

Incubation temperature was observed to have a significant effect on antimicrobial activity. Generally for both *Lactobacillus* strains evaluated, antimicrobial activity of skim milk hydrolysates (10 KDa) obtained by fermentation with *L. acidophilus* NCDC 14 at incubation temperatures of 37 and 42°C was found to be significantly different and was higher at 37°C as shown in Figure 8. Although *L. delbrueckii* NCDC 09 was highly proteolytic but the peptides released by *L. acidophilus* NCDC 14 fermentation had higher antimicrobial activity.

In this study hydrolysis parameters affecting anti-microbial activity that is, proteolytic activity decreased significantly with increased incubation temperature (37 and 42°C) studied (Figure 9). The incubation temperature resulted in significant effect on proteolysis which varied

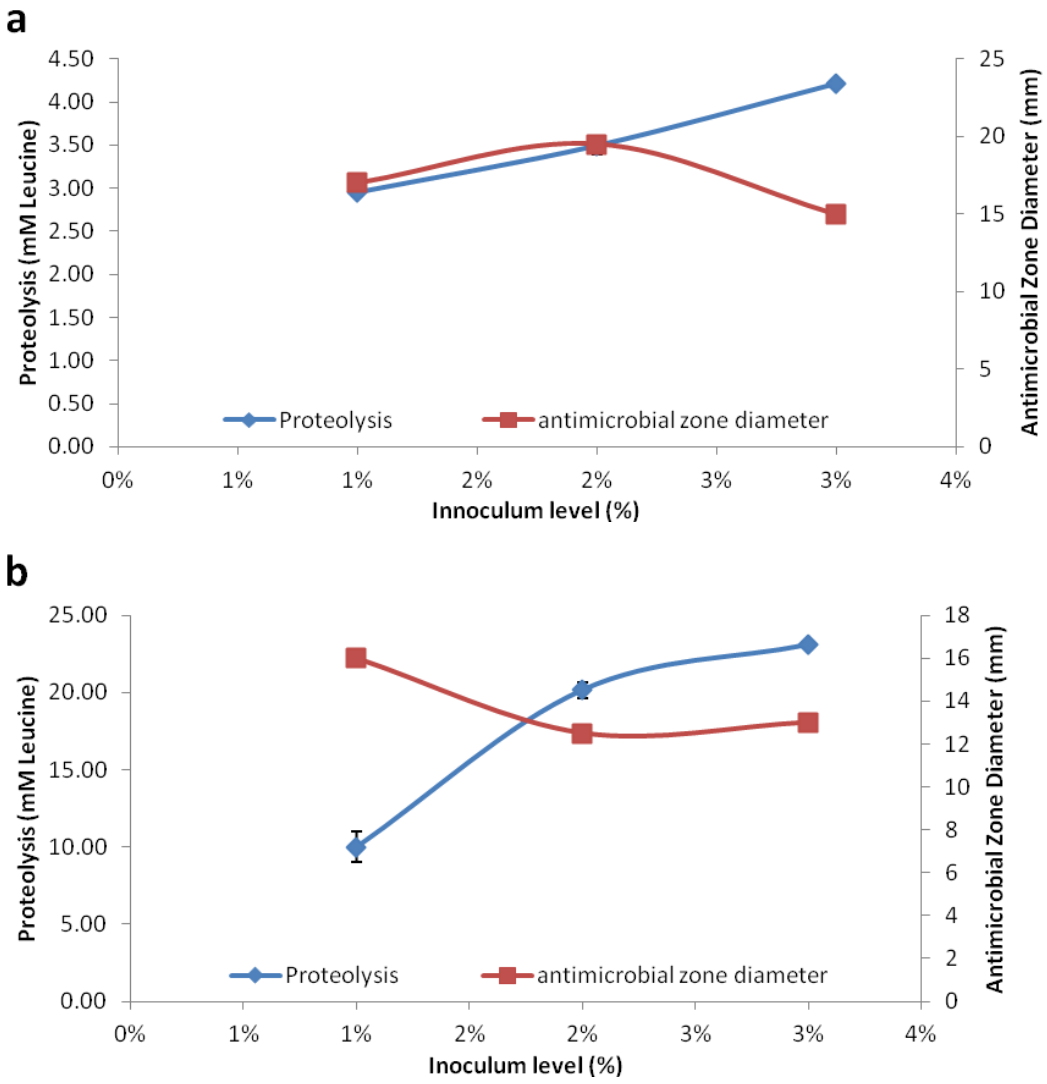

Figure 6. Effect of proteolysis on antimicrobial activity at different inoculums level of *L. acidophilus* NCDC 14 (6a) and *L. delbrueckii* NCDC 09 (6b). Error bars show standard deviation.

among both strains also. The amount of liberated amino groups and peptides vary significantly ($P < 0.05$) during fermentation at two different incubation temperatures. The peptide content was higher than 1 mg/ml at both temperatures but higher degree of proteolysis and peptide content was observed at 37°C for both strains. Like other fermentation variables at two different incubation temperatures, higher proteolysis and peptide content was observed for *L. delbrueckii* NCDC 09 (27 mM Leucine and 5.2 mg/ml) followed by *L. acidophilus* NCDC 14 (6.7 mM Leucine and 2.03 mg/ml) at 37°C followed by 42°C with significant differences ($p<0.05$). Optimum peptide production for both strains was obtained at 37°C. Significant differences were observed in cell concentration and pH decreased at both temperatures. Cell concentration was higher at 37°C and decreased at 42°C for both strains.

DISCUSSION

Results showed that proteinase activity of *Lactobacillus* strains is enhanced in skim milk which is in accordance to previous findings. Hebert et al. (1997) compared proteinase activity of *L. helveticus* CRL 581 cells grown in reconstituted skim milk, MRS and casein-yeast extract glucose (CYG) and reported nine times increase in enzyme activity in milk and about two times in CYG as compared to MRS. Decrease in proteolytic activity might be due to the presence of an available nitrogen source leading to decrease in proteinase activity. As in Lactococci, the production of CEP of *Lactobacillus* was influenced by composition of culture medium; which is in line with the present study (Hebert et al., 2000).

In a number of studies proteinases of *Lactobacillus* species responsible for proteolytic digestion were

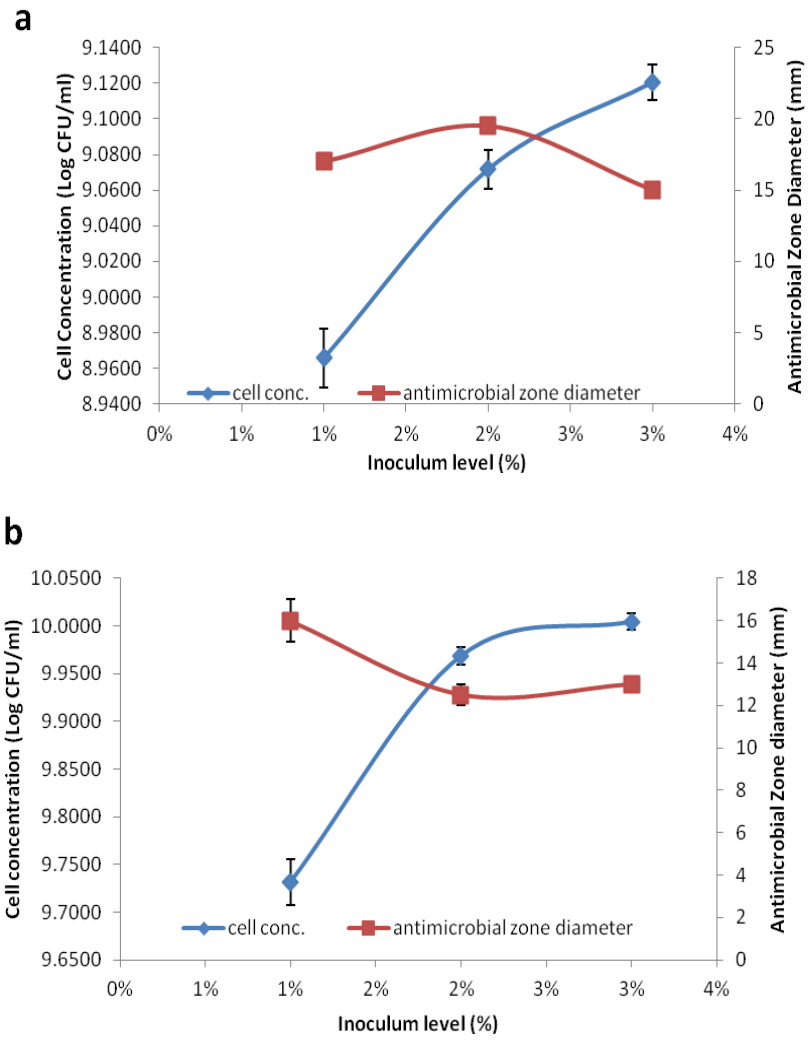

Figure 7. Effect of cell concentration on antimicrobial activity at different inoculum level of *L. acidophilus* NCDC 14 (7a) and *L. delbrueckii* NCDC 09 (7b). Error bars show standard deviation.

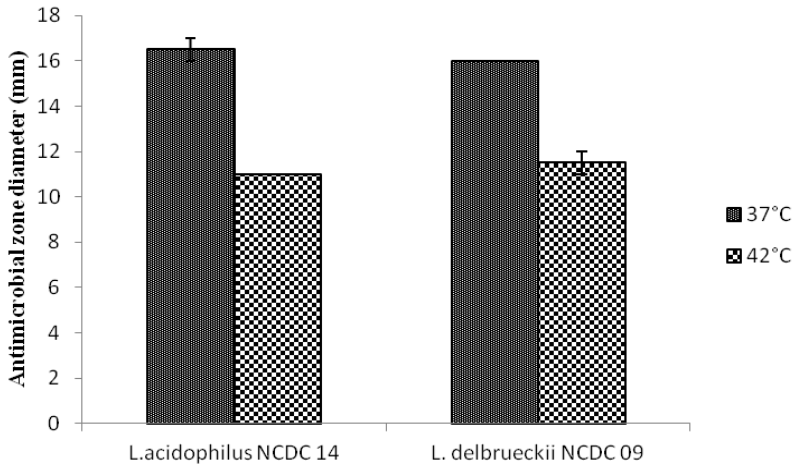

Figure 8. Antimicrobial activity of skim milk hydrolysates fermented by *Lactobacillus* strains at 37 and 42°C. values are the mean±standard deviation (error bars) of three independent experiments.

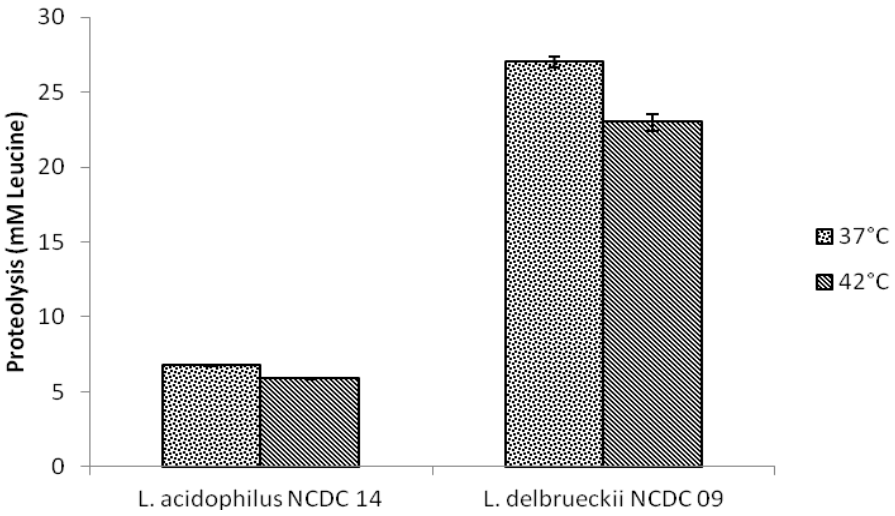

Figure 9. Proteolytic activity of *Lactobacillus* strains at 37 and 42°C. Values are the mean±standard deviation (error bars) of three independent experiments.

studied. In general the degradation of casein is initiated by an extracellular proteinase prtP. Five different types of these enzymes were cloned and characterized from LAB, including PrtP from *L. lactis* and *L. paracasei*, PrtH from *L. helveticus*, PrtR from *L. rhamnosus*, PrtS from *S. thermophilus*, and PrtB from *L. bulgaricus*. LAB typically possesses only one CEP but the presence of two CEPs was reported in *L. bulgaricus*. After the casein-derived peptides are taken up by the LAB cells, they are degraded by a concerted action of peptidases with differing and partly overlapping specificifities. The intracellular endopeptidases, general aminopeptidases (PepN and PepC), and the X-prolyl dipeptidyl aminopeptidase (PepX) are the first enzymes to act on oligopeptides. Other peptidases capable of acting on oligopeptides are the broad-specificity metallopeptidase PepN and cysteine peptidase PepC proteins that were characterized from diverse LAB strains. An enzyme possessing specificity toward di/tripeptides with N-terminal leucine residues and dipeptides containing proline was biochemically characterized from *L. bulgaricus* (Savijoki et al., 2006).

The above stated proteinase and peptidases may produce a number of bioactive peptides among which some may be multifunctional peptides. To date, a repertoire of more than 880 antimicrobial peptides derived from different sources exists in international database (Rydengard et al., 2008). Antibacterial peptides have been derived from milk proteins, lactoferrin, α_{S1}, α_{S2}, k-casein, α lactalbumin, β- lactoglobulin, and lysozyme. Caseicins A, B and C, isracidin α_{S1}f(1-23), Casocidin-I α_{S2}-CN(150-188), CMP k-casein–A, f(138-158), k-casecidin k-CN f(17-21), Lactoferricin-H lf(1-47), lactoferrampin lf(268-284) are different known antimicrobial peptides. Along with these peptides another novel peptides may also appear in the present study depending on specifities of proteinases and peptidases of both strains.

The ability of *Lactobacillus* strains to generate bioactive peptides during milk fermentation was observed to be a strain specific characteristic (Pihlanto et al., 2010) which might be connected to many factors such as bacterial growth, organic acid production and proteolytic activity of these strains. Furthermore, the time-dependent release of various peptides observed in our study might have important consequences on the extent of *in vitro* antimicrobial activity in fermented milk, which deserves further elaboration. The antimicrobial activity pattern observed for both strain could be associated with the proteolytic activity of strains and its ability to produce antimicrobial peptides stronger than others (Fuglsang et al., 2003). Our results are in accordance with Anas et al. (2008) who evaluated antimicrobial activity of 8 Lactobacillus species of three genera *L. plantarum*, *L. casei* and *L. rhamnosus* which increased with the incubation period and reduced the growth of *Staphylococcus aureus* by 1.6 log phase within 24 h and no bacteria was found after 72 h.

During fermentation, milk proteins were hydrolysed by LAB proteinases and peptidases resulting in an enhanced amount of free amino groups and peptides. The extent of proteolysis varied among both strains and appeared to be time dependant. Leclerc et al. (2002) demonstrated a linear increase in extent of proteolysis with fermentation time for *L. helveticus* as observed in present study. In a different study protease activity increased with incubation period up to 36 h in MRS and decreased thereafter being highest for *L. rhamnosus* followed by *L. delbrueckii*, *L. helveticus* and *L. plantarum* (Kholif et al., 2011). Haq and Muktar (2009) reported

maximum proteolytic activity of 15 Lactobacillus strains after 48 h of cultivation which corresponded to logarithmic growth phase of the bacterium and thereafter steady decrease in amount of protease.

The proteolysis further may be dependent on growth performance of the strains which may also affect the antimicrobial peptide production. For *L. delbrueckii* NCDC 09 the drop of pH was much faster which is in concordance to the Heller (2008) that *L. delbrueckii* ssp. *bulgaricus* leads to higher decrease in pH as compared to *L. acidophilus*, *L. brevis* and *L. casei* when used as starter organisms for dairy products. Kholif et al. (2011) reported increase in log count up to 48 h with simultaneous decrease in pH by strains that *L. casei*, *L. acidophilus* and *L. delbrueckii*. Cell concentration has great effect on proteolysis as well as antimicrobial activity as depicted in our study and also reported by many previous studies (Ramesh, 2011; Franca et al., 2009).

For high bio-product synthesis inoculums size must be controlled to ensure optimum nutrient uptake in a culture medium. The inoculum size resulted in significant effect on antimicrobial activity which varied among strains also as higher inoculum level reduced the antimicrobial activity. Tadesse et al. (2006) reported higher growth of LAB in MRS broth (a complete nutrient media) led to higher acid production and metabolites with antimicrobial property while LAPTg broth with inferior nutrition caused lower cell concentration with higher antimicrobial activity. Thus higher number of bacteria produced may have adverse effect on bioactivity. In contrast Misra and Kuila (1992) reported same antibacterial activity at 5, 10 and 15% inoculum levels against four organisms that is *E. coli*, *Shigella dysenteriae*, *Staphylococcus aureus* and *Bacillus cerus*.

The inoculum size resulted in significant effect on proteolytic activity which varied among strains also and with increasing inoculums level (1-3%) proteolytic activity increased. Small inoculum size as 1% level for *L. acidophilus* NCDC 14 means insufficient number of bacteria secreted reduced amount of protease leading to lower hydrolysis. An increase in inoculum density led to higher biomass productivity with higher hydrolysis but lower bioactivity (Danquah and Forde, 2007) which agrees with present study also. However in another study Agyei et al. (2012) reported at higher cell densities (>5%) growth rate of *L. delbrueckii* sub sp. Lactis ATCC 7830 decreased with lower CEP production due to increasing limitation of key nutrients. An increase in inoculum density usually leads to higher biomass productivity but lower average specific growth rate (Koutsoumanis and Sofos, 2005).

Temperature is an important environmental factor affecting all the physiological activities in a living cell and controls the growth, microbial activities, and normal functioning of the cellular enzymes. Antimicrobial activity of *Lactobacillus* strains was higher at 37°C than 42°C might be due to higher growth rate. In a similar study for development of Bifidus milk, Misra and Kuila (1992) studied effect of incubation temperature on antibacterial activity. At 32°C and 40°C no zone of inhibition was observed against *Shigella dysenteriae*, *E. coli* and *Staphylococcus aureus* while at 37°C good inhibition zone was obtained. The optimum temperature for growth of *B. bifidum* was 37°C and maximum production of antimicrobial substance was expected at this temperature as in the present study also optimum peptide production for both strains was obtained at 37°C (data not shown).

With decreasing proteolysis at 42°C (Figure 9) antimicrobial activity has also decreased as suggested by positive correlation coefficient (r^2=1). The sharp decrease in proteolytic and antimicrobial activity at 42°C may be due to thermal inactivation of biosystems at temperature higher than the optimum (Ageyi et al., 2012). Increasing limitation of key nutrients and accumulation of growth inhibitory metabolites or maximum cell lysis when fermentation leads to maximum growth rate could be another cause of decrease in cell concentration at 42°C reported by Otte et al. (2011) for *L. helveticus*. Although *L. delbrueckii* NCDC 09 was highly proteolytic but the peptides released by *L. acidophilus* NCDC 14 fermentation had higher antimicrobial activity. In a similar study higher proteolytic bacteria *L. casei* did not show higher ace-inhibitory activity which means higher proteolysis may not lead to higher bioactivity (Donkor et al., 2007). Furthermore bioactivity is also strain dependent as Fugalsang et al. (2003) reported varying amounts of ace-inhibitors produced by most LAB during milk fermentation varied with strains.

Corelation between proteolysis and antimicrobial activity

As shown in Figure 3a and b proteolysis was highest at 48 h for *L. acidophilus* NCDC 14 and at 24 h for *L. delbrueckii* NCDC 09. At different incubation periods positive correlation (r^2=0.99, 0.64) was observed between proteolysis and antimicrobial activity for both strains. With increasing proteolysis antimicrobial activity increased but after optimum incubation period antimicrobial activity decreased with increasing proteolysis. As shown in Figure 6a and b increasing inoculums level increased the proteolysis but not antimicrobial activity. Higher proteolysis lysed antimicrobial peptides leading to loss of activity and other new peptides to appear (Donkor et al., 2007). For *L. delbreuckii* NCDC 09 higher antimicrobial zone diameter observed at lower inoculum level (1%) can explain for negative correlation (r^2= -0.94) among proteolysis and antimicrobial activity. Similarly for *L. acidophilus* NCDC 14 antimicrobial activity increased only up to 2% level in contrast to 3% for proteolysis. The results are in agreement with the study of Pan and Guo (2010) that is ace-inhibitory activity released during the first hydrolysis reaction time and further digestion did not

Table 1. Optimum conditions of fermentation for generation of antimicrobial peptides by *L. acidophilus* NCDC 14 & *L. delbrueckii* NCDC 09.

Strain	Parameter	Antimicrobial activityzone dia. (mm)	Peptide content (mg/ml)
	48 h	20 ± 1	2.25±0.003
L. acidophilus NCDC 14	2%	19.5 ± 0.5	1.79±0.10
	37°C	16.5 ± 0.5	2.03±0.1
	24-36 h	15±2, 16.5 ± 1.5	4.2±0.06-5.19±0.04
L. delbrueckii NCDC 09	1%	16 ± 1	3.50±0.28
	37°C	16 ± 0	5.20±0.15

increased activity. Thus proteolytic activity that affects the release of various peptides observed in this study might have important consequences on the extent of antimicrobial activity in fermented milk.

Optimized conditions for generation of antimicrobial peptides by selected *Lactobacillus*

Fermented milks showed good antimicrobial activity with significant differences among them. Incubation period, inoculation volume and incubation temperature showed significant effect on antimicrobial activity. Both strains showed higher antimicrobial activity at different incubation periods that is *L. acidophilus* in 48 h, *L. delbrueckii* NCDC 09 in 12-24 h which could be correlated to proteolysis and peptide content as activity increased and then decreased at a particular incubation periods. With proteolysis in range of 7.34-8.5 mM Leucine and 20-27.1 mM Leucine *for L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09 respectively antimicrobial activity increased (17 mm< and 15 mm<) and above this stated range activity decreased. *L. acidophilus* and *L. delbrueckii* NCDC 09 showed higher activity at different inoculum level that is 2 and 1% respectively. Both strains showed good antimicrobial activity at 37°C which seemed to be optimal temperature for growth. Optimized conditions for antimicrobial peptides generation with peptide content by selected *Lactobacillus* are presented in Table 1.

Conclusion

This work has shown, for the first time, that incubation period (48 and 12-24 h), inoculum size (2 and 1%) incubation temperature (37°C) is important variables determining the production of antimicrobial peptides from skim milk by *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09. The optimal condition yielded 2.43-2.90 mg/ml and 20-27 mg/ml peptides from skim milk by *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09 fermentation respectively. The usefulness and application of antimicrobial peptides in coming years, is likely to expand beyond their medicinal properties as well as their use in food. Detailed optimization of fermentation parameters is essential for the generation of peptides at optimum process and economic conditions. The results of this study give useful information towards the achievement of such ends, in addition to providing and extending utility of antimicrobial peptides from skim milk by *L. acidophilus* NCDC 14 and *L. delbrueckii* NCDC 09 fermentation.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Polyhydroxybutyrate production by *Spirulina* sp. LEB 18 grown under different nutrient concentrations

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In response to the environmental problems caused by plastics of petrochemical origin, a reduction in the use of these materials and their replacement by biodegradable polymers have been sought. Polyhydroxybutyrate (PHB), a biopolymer of biological origin that belongs to the polyhydroxyalkanoates (PHAs), is similar to polypropylene in terms of its mechanical properties, thermodegradability and melting temperature. Various microorganisms, including cyanobacteria, can synthesize this biopolymer. The objective of this study was to stimulate biopolymer synthesis by *Spirulina* sp. LEB 18 that was grown under different nutritional conditions. Initially, the growth was conducted with *Spirulina* sp. LEB 18 without the adaptation of the inoculum. In these assays, the concentrations of the carbon, nitrogen and phosphorus sources were varied. The assay that showed the maximum concentration of biopolymers was reproduced with the adaptation of the inoculum for 45 days. There was an inverse relationship between the cell growth and biopolymer synthesis. The assay that contained 0.25 g L^{-1} sodium nitrate, 4.4 g L^{-1} sodium bicarbonate and 0.5 g L^{-1} potassium phosphate showed the maximum cell concentration (0.6 g L^{-1}) and a low biopolymer accumulation (13.4%). In addition, the assay that contained 0.05 g L^{-1} sodium nitrate, 8.4 g L^{-1} sodium bicarbonate and 0.5 g L^{-1} potassium phosphate produced a high biopolymer concentration (30.7%) and a low cell concentration (0.5 g L^{-1}). The adaptation of the inoculum increased the cell concentration by 7.0% and the biopolymer yield by 20.5%. The biopolymer production was more efficient in assays in which the nitrogen was restricted and had maximum carbon consumption.

Key words: Biopolymers, polyhydroxyalkanoates, microalgae, cyanobacteria.

INTRODUCTION

Over the past 50 years, plastics of petrochemical origin have been used in many applications. Plastics are an indispensable part of many industries, having replaced glass and paper packaging over the years (Khanna and Srivastava, 2005). Their versatility, technical properties and cost (1 kg of polypropylene costs approximately US $ 1.00) have led to their widespread use.

With the forecasted reduction in oil reserves and the

biological non-degradation nature of these materials, sources for alternatives to petrochemical plastics are receiving a large amount of attention (Panda et al., 2006). Polyhydroxyalkanoates (PHAs) are biopolymers, their thermal degradation characteristics, melting temperature and mechanical properties are similar to polypropylene and can act as an alternative to plastics of petrochemical origin (Bugnicourt et al., 2014). Polyhydroxybutyrate (PHB) is the most studied representative of the PHA group. PHB also presents characteristics such as biodegradability, thermoplasticity, processibility, hydrophobicity and biocompatibility with cells and tissues, which suggests attractive applications in the food, pharmaceutical and medical areas (Sharma and Mallick, 2005). When broken down by microorganisms, PHAs form water and CO_2, which can be reintegrated into nature and close the carbon cycle. These polyesters are produced by various prokaryotic microorganisms, such as cyanobacteria (Balaji et al., 2013).

Cyanobacteria are photoautotrophic aerobes that form part of the microalgae group. Their vital processes require water, carbon dioxide, and inorganic light. Photosynthesis is the main way to obtain energy by converting nutrients in the medium to cellular material and releasing oxygen to the environment (Pelizer et al., 2003). The *Spirulina* is a photosynthetic cyanobacteria and producer of various products such as biofuels and biopolymers (Nautiyal et al., 2014; Jau et al., 2005). The strain *Spirulina* sp. LEB 18 is studied since 2008 when it was isolated. Since then several studies have been conducted with this micro-organism, such as biomass, dyes, biosurfactants, biofuels, biopolymers and effluent treatment (Morais et al., 2015).

The search for alternatives for the production of biodegradable plastics involves the search for new processes and materials while making use of biotechnology, through the use of microorganisms and their metabolic products. The knowledge of these microorganisms is very important in the conversion of certain substances into others and in the possible use of substrates in obtaining viable products and by-products.

The substrate conversion efficiency is important and depends on the physiology and biochemistry involved in the synthesis of biopolymers. Among the various nutrients in the medium, the carbon source contributes more significantly to the overall cost of the production process and to the stimulation of the biopolymer synthesis. Nitrogen and phosphorus are also important in PHB synthesis and should be limited in such a way that the metabolic pathway of the microalgae is directed to the production of biopolymers and not other bioproducts. Thus, the objective of this study was to stimulate the production of biopolymers by *Spirulina* sp. LEB 18 by varying the nutritional conditions.

MATERIALS AND METHODS

Growth of *Spirulina* sp. LEB 18 without adaptation of the inoculum

In this study, *Spirulina* sp. LEB 18 isolated from Mangueira Lagoon (33º30'12"S, 53º08'58"W) was used. Zarrouk medium was used to maintain the inoculum of *Spirulina* sp. LEB 18 (Table 1) (Zarrouk, 1966).

The cultures were grown in 2.0 L Erlenmeyer type photobioreactors with a working volume of 1.8 L. The initial biomass concentration of the assay was 0.15 g L^{-1}, maintained at 30°C in a thermostated chamber in a single batch process. The illuminance was 59.2 µmol m^{-2} s^{-1}, with a 12 h light / 12 h dark photoperiod. The assays lasted 15 days, and at the end of the growth, the extraction of the biopolymers of microalgal biomass was conducted. The carbon ($NaHCO_3$), nitrogen ($NaNO_3$) and phosphorus (K_2HPO_4) sources of the Zarrouk culture medium were evaluated in three concentrations (Table 2).

Growth of *Spirulina* sp. LEB 18 with adaptation of the inoculum

The inoculum of *Spirulina* sp. LEB 18 used in this assay was kept for 30 days with 8.4 g L^{-1} of sodium bicarbonate, 2.5 g L^{-1} sodium nitrate and 0.5 g L^{-1} potassium phosphate. After this period, the inoculum was diluted with Zarrouk with no carbon, nitrogen and phosphorus source in a ratio of 1:1 and cultured for 15 more days. After 45 days of adaptation of the inoculum, the assay with concentrations of carbon, nitrogen and phosphorus selected as the best biopolymer producer (assay 7: 8.4 g L^{-1} sodium bicarbonate, 0.05 g L^{-1} sodium nitrate, and 0.5 g L^{-1} potassium phosphate) was started. After the adaptation, the assays were started in the same conditions of 2.1 item.

Monitoring of the growth and responses

Samples were collected aseptically every 24 h to monitor the cell concentration, which was calculated by measuring the optical density at 670 nm in a spectrophotometer (Q7980RM, Quimis, Brazil), with a calibration curve that relate the optical density and dry biomass for each microalgae (Martins et al., 2014). The pH of the cultures was monitored daily with a digital pH meter (pH221, Lutron, Brazil).

From the data on the cell concentrations, the kinetic parameters of the microalgae growth and biopolymer yield (ηbiopol, %) were determined. The maximum specific growth rate (μ_{max}, d^{-1}) was calculated by exponential regression of the log phase of the cell growth curve.

The generation time (tg, d) is the time required to double the biomass. The maximum cell concentration is the maximum concentration obtained. The biomass yield (P, g L^{-1} d^{-1}) is defined as the mass formed in a given volume per unit of time. P_{max} is the

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Table 1. Composition of the Zarrouk culture medium.

Reagent	Quantity
$NaHCO_3$	16.8 (g L^{-1})
K_2HPO_4	0.5 (g L^{-1})
$NaNO_3$	2.5 (g L^{-1})
K_2SO_4	1.0 (g L^{-1})
NaCl	1.0 (g L^{-1})
$MgSO_4.7H_2O$	0.2 (g L^{-1})
$CaCl_2$	0.04 (g L^{-1})
$FeS0_4.7H_2O$	0.01 (g L^{-1})
EDTA	0.08 (g L^{-1})
Solution A5	1 mL
Solution B**6**	1 mL

Solution A_5: 2.86 g L^{-1} of H_3BO_3; 1.81 g L^{-1} of $MnCl_2.4H_2O$; 0.222 g L^{-1} of $ZnSO_4.7H_2O$; 0.079 g L^{-1} of $CuSO_4$. $5H_2O$; 0.015 g L^{-1} of $NaMoO_4$. Solution B_6: 22.96 mg L^{-1} of NH_4VO_3; 96 mg L^{-1} of $K_2Cr_2(SO_4)_4.24H_2O$; 47.85 mg L^{-1} of $NiSO_4.7H_2O$; 17.94 mg L^{-1} of $Na_2WO_4.2H_2O$; 61.1 mg L^{-1} of $TiOSO_4.H_2SO_4.8H_2O$; 43.98 mg L^{-1} of $CO(NO_3)_2.6H_2O$.

maximum productivity value found during growth. The biopolymer yield was obtained by the dry weight ratio of the microalgal biomass and the dry mass of the biopolymer extracted.

Carbon, nitrogen and phosphorus consumption during growth

The carbon concentration in the medium was determined by analysis of the alkalinity (ASTM, 2011). The nitrogen concentration was determined using the colorimetric method, where 10.0 mL of the sample is centrifuged and 0.2 ml of the supernatant is removed. Next, 0.8 mL of sulfuric acid with salicylic acid reagent was added, and after 20 min, 19.0 ml of 2N NaOH was added. After cooling, the absorbance was determined at 410 nm. Through using a standard curve, which was accomplished prior to the assays, the nitrogen concentration results were found in the form of nitrate. From stoichiometric calculations, the molecular nitrogen concentration (g L^{-1}) in the culture medium (Cataldo et al., 1975) was determined.

The phosphorus concentration was determined by phosphate analysis using a colorimetric kit (PhosVer 3, Hach, USA). In this analysis, a 10 mL sample of the previously diluted medium is placed in contact with the phosphate Kit, and after 2 min, a reading of the absorbance is conducted at 890 nm. Through the standard curve, the phosphorus concentration (g L^{-1}) was determined and given in molecular phosphorus form. Carbon, nitrogen and phosphorus were analyzed after 0, 5, 10 and 15 days of culture.

Extraction of biopolymer by digestion

The microalgal biomass was centrifuged at 7500 rpm for 20 min at 20°C, and the supernatant was then discarded. The precipitate obtained in the centrifugation was stirred for 10 min with sodium hypochlorite (10.0%, v/v) and distilled water to give a final concentration of 4.0% (v/v). The centrifugation and stirring with sodium hypochlorite steps were repeated. The precipitate was then washed with distilled water, centrifuged and washed with acetone while stirring for 10 min. The precipitate was centrifuged to separate the liquid and taken to the oven (Quimis, Q317 M, Brazil) at 35°C for 48 h. The obtained biopolymer was then weighed (Martins et al., 2014).

Characterization of the extracted biopolymer

The samples were characterized for the initial degradation temperature (TD_0, °C), final degradation temperature (TD_f °C) and non-degraded material (IP, % w/w) by differential thermogravimetric analysis (DTG) (DTG-60, Shimadzu, Japan). Approximately 5.0 mg of the sample was heated from 25 to 500°C at 10°C min^{-1} under a nitrogen atmosphere. The initial and final temperatures of the degradation were determined by the first derivative of the DTG curves, and the degree of the non-degraded material was considered to be the amount of sample that remained at the end of the analysis.

RESULTS AND DISCUSSION

The assays performed without the adaptation of inoculum as well as those with prior adaptation showed cell growth during the 15 days of growth, with no lag phase and cell decline observed. The phase adjustment or lag phase is the period in which the cell synthesizes the enzymes necessary for the use of the components present in the medium. If the cells are pre-adapted to the environmental conditions of the assay, then the length of time of the induction phenomenon can be reduced or does not exist. In these assays, although the nutrient concentration is changed, the sodium nitrate, sodium bicarbonate and potassium phosphate used in the assays are the same sources of nitrogen, carbon and phosphorus as in the Zarrouk culture medium, respectively. Thus, *Spirulina* sp. LEB 18 showed no lag phase because it did not need to develop differentiated structures to metabolize the nutrients.

Among the assays performed without adaptation of the inoculum, it was observed that assay 7 (0.05 g L^{-1} $NaNO_3$, 8.4 g L^{-1} of $NaHCO_3$ e 0.5 g L^{-1} of K_2HPO_4) showed a maximum biopolymer yield (30.7%). In assay 7, a lower growth trend was observed, followed by assay 9, which had the second highest biopolymer yield (22.7%) (Figure 1). Assay 6 showed a growth curve that was superior to the others after 10 days of culture (Figure 1), reaching the final 15 days with a maximum cell concentration of (0.6 g L^{-1}) and biopolymer yield of 13.4% (Table 3).

From the growth curves in Figure 1 and Table 3, it was observed that the tests where the cell concentration is maximum, the yield of PHB was minimal. The same behavior was observed by Samantaray et al. (2011), when growing the cyanobacterium *Aulosira fertilíssima* to obtain PHB.

The maximum specific growth rate was between 0.07 d^{-1} (assay 8) and 0.1 d^{-1} (assay 1). Assay 8 was conducted with the maximum values for the carbon, nitrogen and phosphorus sources, while assay 1 showed the minimum

Table 2. Concentrations of nitrogen, carbon, phosphorus and C/N ratio added in the assays performed with *Spirulina* sp. LEB 18.

Assay	$NaNO_3$(g L^{-1})	$NaHCO_3$(g L^{-1})	K_2HPO_4(g L^{-1})	C/N
1	0.05	4.4	0.1	78
2	0.25	4.4	0.1	16
3	0.05	8.4	0.1	149
4	0.25	8.4	0.1	30
5	0.05	4.4	0.5	78
6	0.25	4.4	0.5	16
7	0.05	8.4	0.5	149
8	0.25	8.4	0.5	30
9	0.15	6.4	0.3	38
7a*	0.05	8.4	0.5	149

*Assay 7 with adaptation of the inoculum

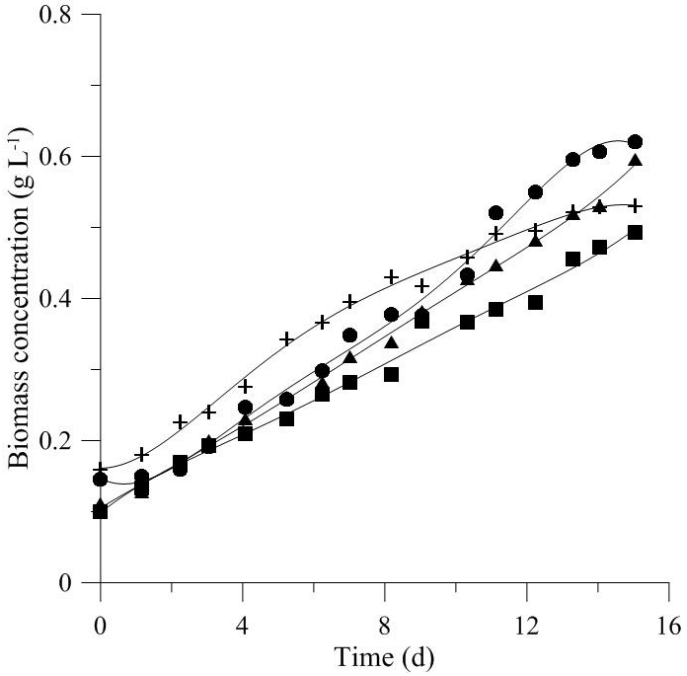

Figure 1. Cell concentration as a function of time for *Spirulina* sp. LEB 18 grown without adaptation of the inoculum. ● 0.25 g L^{-1} of $NaNO_3$, 4.4 g L^{-1} $NaHCO_3$ and 0.5 g L^{-1} K_2HPO_4 (Assay 6), ■ 0.05 g L^{-1} of $NaNO_3$, 8.4 g L^{-1} $NaHCO_3$ and 0.5 g L^{-1} K_2HPO_4 (Assay 7), ▲ 0.15 g L^{-1} of $NaNO_3$, 6.4 g L^{-1} $NaHCO_3$ and 0.3 gL^{-1} K_2HPO_4 (Assay 10), and + *Spirulina* sp LEB 18 grown with adaptation of the inoculum containing (+) 0.05 g $L^{-1}$of $NaNO_3$, 8.4 g L^{-1} $NaHCO_3$ and 0.5 g L^{-1} K_2HPO_4.

values. The minimum generation time was 5.9 days (assay 8). The minimum X_{max} found was 0.4 g L^{-1} in assay 9, which showed a low carbon consumption (2.4%, Table 3) and P_{max} (0.02 g L^{-1} d^{-1}). The low carbon consumption of assay 9 could have hampered the growth of the microorganism. Carbon is one of the most important chemical elements for the growth of the microorganisms and is responsible for the formation of organic nutrients of the 3 major classes: carbohydrates, proteins and lipids. These compounds provide energy for cell growth. A typical cell consists of approximately 50.0% carbon, which is the principal component of all of the classes of macromolecules (Markou et al., 2014).

The highest yield was observed in assay 7 (0.04 g L^{-1} d^{-1})

Table 3. Maximum cell concentration ($X_{max,}$ g L-1), maximum yield (P_{max}, g L^{-1} d^{-1}), maximum specific growth rate (μ_{max}, d^{-1}), generation time (tg, d) and biopolymer yield (ηbiopol, %) on day 15 for *Spirulina* sp. 18 LEB grown with different concentrations of sodium nitrate, sodium bicarbonate and potassium phosphate.

Assay	X_{max}	P_{max}	μ_{max}	tg	η_{biopol}
1	0.4	0.03	0.07	10.0	14.1
2	0.4	0.03	0.07	9.6	17.3
3	0.5	0.03	0.08	8.9	16.9
4	0.5	0.03	0.08	8.2	14.7
5	0.5	0.03	0.08	8.8	13.4
6	0.6	0.03	0.10	6.7	13.4
7	0.5	0.04	0.10	7.1	30.7
8	0.6	0.03	0.12	5.9	16.3
9	0.4	0.03	0.11	6.5	22.7
7a	0.5	0.03	0.07	8.6	38.6

followed by growth with the same concentration of carbon, nitrogen and phosphorus with adaptation of the inoculum (0.03 g L^{-1} d^{-1}). Comparing assay 7 with the others, it was found that the growth was conducted with the smallest maximum concentration of nitrogen, carbon (higher C/N tested) and maximum phosphorus. The microorganisms respond to nitrogen limitation by the preferential degradation of one or more macromolecules that contain this component, which results in the reduction of these nutrients and the accumulation of carbon reserve compounds such as lipids, including PHAs. The phosphorus component is essential to the metabolism of microalgal having direct influence on the formation of organic molecules such as nucleic acids (DNA and RNA), membrane phospholipids and ATP (Markou et al., 2014).

When repeating the conditions of assay 7 but performing adaptation of the inoculum for 45 days, an increase of 7.0% in the cell concentration and 20.5% in the yield of the biopolymers was observed. When Panda et al. (2006) conducted an inoculum adaptation of *Synechocystis* sp. PCC 6803 with glucose and an addition of 1.5 g L^{-1} of $NaNO_3$, 0.04 g L^{-1} K_2HPO_4 and 0.02 gL^{-1} of Na_2CO_3 in 150 ml flasks with 50 ml BG11 culture medium, at a temperature of 28°C with a 14 h light and 10 h dark photoperiod, they observed an increase of 29.0% in the PHB accumulation.

From the growth curves (Figure 1), it was observed that the inoculum-adapted assay had a higher development than the others until the 10th day of growth. After this period, the microalgae reduced its growth, finalizing the growth deceleration phase and entering the stationary phase. The deceleration phase occurs from the exhaustion of one or more components of the culture medium necessary for growth and from the accumulation of inhibitory metabolites. The instantaneous and specific growth velocities decrease until they cancel out each other at the end of the time (t_f), which is when the stationary phase begins.

This reduction in growth after 10 days occurred in parallel to the limitation of nitrogen that had already been 100% consumed at this time (Table 4). Thus, the microorganism reduced the biomass production by the limitation of the source of the nitrogen, and the biopolymer synthesis was stimulated, which occurred in assay 7a, where the maximum value was found (38.6%).

To better understand the PHB synthesis process by cyanobacteria, we will divide into three stages: photosynthesis, tricarboxylic acid cycle (TCA) and the synthesis of PHB. In the first stage, light energy is collected by breaking water molecules and releasing O_2, ATP, NADPH and glyceraldehyde-3-phosphate. It is suffering reactions to yield acetyl-CoA. The second stage is responsible for the use of acetyl-CoA in the formation of carbon skeletons and energy generation. The third step is the synthesis of PHB, which starts by the condensation of two acetyl-CoA molecules by the enzyme β-ketothiolase give acetoacetyl-CoA. This in turn is reduced to (R) -3-hydroxybutyryl-CoA reaction catalyzed by the enzyme 3-ketoacyl-CoA reductase NADPH dependent. The formation of PHB is terminated by polymerizing two or more molecules of (R) -3-hydroxybutyryl-CoA by the enzyme catalyzed reaction PHB synthase (Wang et al., 2013).

Then under balanced growth conditions, where all of the nutrients necessary for cell multiplication are available, the levels of free co-enzyme A (CoA) are high. Thus, it is possible to meet the great demands of the acetyl groups with the Krebs cycle, for the formation of carbon skeletons and energy generation. Free CoA has an inhibitory effect on the enzyme β-ketothiolase, which prevents the synthesis of PHB. When a certain nutrient becomes limiting to the multiplication of the microorganism, the demand for acetyl-CoA decreases. Thus, the

Table 4. Nitrogen concentration at time zero (X_{NO}, mg L^{-1}), nitrogen concentration at time 15 d (XN_{15}, mg L^{-1}), nitrogen consumption after 15 days (C_{N15}, % w/w), carbon concentration at time zero (X_{C0}, g L^{-1}), carbon concentration at time 15 days (X_{C15}, g L^{-1}), carbon consumption after 15 days (C_{C15}, % w/w), phosphorus concentration at time zero (X_{PO}, g L^{-1}), phosphorus concentration at time 15 days (X_{P15}, g L^{-1}) and carbon consumption after 15 days (C_{P15}, % w/w) in the *Spirulina* sp. 18 LEB assays.

Assay	X_{N0}	X_{N15}	C_{N15}	X_{C0}	X_{C15}	C_{C15}	X_{P0}	X_{P15}	C_{P15}
1	15.7	0.8	94.7	0.4	0.3	15.0	0.10	0.07	30.0
2	61.5	1.7	97.2	0.5	0.4	17.8	0.10	0.07	30.0
3	14.9	0.2	98.4	1.2	1.0	19.0	0.10	0.07	30.0
4	61.6	0.7	98.7	1.3	1.0	21.7	0.10	0.07	30.0
5	13.0	Nd	100	0.5	0.4	17.6	0.49	0.14	71.4
6	53.1	0.4	99.3	0.4	0.4	4.5	0.50	0.15	70.0
7	12.3	Nd	100	1.2	0.9	17.2	0.50	0.13	74.0
8	52.2	0.02	99.9	1.2	1.1	6.5	0.50	0.14	72.0
9	21.9	Nd	100	0.4	0.4	2.4	0.33	0.09	72.7
7a	15.7	Nd	100	1.2	0.6	50.0	0.50	0.29	42.6

*Nd: Undetectable.

free CoA level becomes reduced, decreasing the inhibition of the β-ketothiolase and triggering the synthesis of PHB (Laycock et al., 2013).

The process of reducing acetoacetyl-CoA to (R)-3-hydroxybutyryl CoA by 3-ketoacyl-CoA reductase enzyme is NADPH dependent, relationship between NADP and NADPH is necessary for the reaction. As previously mentioned, this relationship is maintained by photosynthesis, which produces O_2 and NADPH. It is understood then that for PHB accumulation of NADPH/ NADP ratio should be high, as observed by Hauf et al. (2013), which monitor the presence of NADP and NADPH in the cells of Synechocystis PCC6803 to obtain PHB obtained as the best response to relative high NADPH/NADP. The authors, as in this study, have obtained high C/N to be the best for obtaining PHB.

In the inoculum-adapted assay, biopolymer extraction was conducted during 5, 10 and 15 days of assays, obtaining a 25.2, 40.9 and 38.6% yield, respectively. The maximum biopolymer yield (40.9%) obtained at day 10 could be due to the microorganism being at the end of the deceleration phase and in the early stationary phase. In the deceleration phase, the microorganism reduces its growth and therefore can target its metabolism to bioproduct synthesis, in this case the biopolymers.

Sharma and Mallick (2005) grew the microalgae *Nostoc muscorum* for 42 days, periodically monitoring the PHB accumulation. The authors obtained a maximum PHB yield (8.6%) at day 21 of the growth, which is similar to that obtained in this study, at the end of the deceleration phase and in the early stationary phase. Panda et al. (2006) obtained a maximum PHB accumulation in the stationary phase (4.5 w/w) compared to the lag (1.8%) and Log (2.9%) phases.

In this study with *Spirulina* sp. LEB 18, there was a reduction in the concentration of the polymer after 15 days of growth compared to day 10. This reduction occurred because during the stationary phase, there is a balance between the growth rate and death rate of the microorganism as well as biochemical changes in the cell structure that stop the synthesis of the byproducts. According to Sharma and Mallick (2005), after the *Nostoc muscorum* reached the maximum biopolymer yield in the early stationary phase, there was a reduction in the buildup of the byproduct. The authors explain that this reduction is due to the use of PHB as a carbon source by the microorganism.

At the beginning of the growth, the pH of the assays without inoculum adaptation presented values between 9.9 and 10.3, and after 15 days, these values were 10.5 and 10.7. The minimum observed was pH 9.8 at day 3 of the culture (Assay 9). Assay 8 showed the maximum pH value (10.8), which was observed on day 15. According to Sharma and Mallick (2005), the maximum accumulation of PHB in the culture with the microalgae *Nostoc muscorum* grown in the BG-11 medium for 21 days was 8.9% (w/w) at pH 8.5, followed by 7.8% (w/w) at pH 9.5 and 7.2% at pH 10.5. In acidic pH, the authors did not have detectable amounts of PHB.

Assays 1, 2, 5 and 6, which had 4.4 g L^{-1} sodium bicarbonate solution, showed stable pH during the whole growth period. With the exception of assay 6, cultures 1, 2 and 5 showed pronounced consumption of carbon (15.0 and 17.8%), which justifies the pH stability (Table 4). In cultures that had higher concentrations of sodium bicarbonate (6.4 and 8.4 g L^{-1}), the pH increased by approximately 0.7 units.

The inoculum-adapted culture showed a pH that was superior to that of assays without adaptation, which ranged from 10.2 to 11.1, with a value of 10.3 on the first

day of growth and 10.4 on the last day. Sodium bicarbonate has a buffering action, which causes the pH to remain stable. The inoculum-adapted culture had the highest carbon source consumption (50.0%) (Table 4) and high pH stability compared to the other assays.

The assays conducted without inoculum adaptation presented nitrogen consumption between 94.1 and 100% (Table 4). With the maximum biopolymer yield (30.7%) compared to cultures without adaptation of the inoculum, assay 7 showed 100% nitrogen consumption before the 5th day. Panda et al. (2006) obtained 9.5% PHB in *Synechocystis* sp. PCC 6803, grown under a nitrogen limitation.

Assay 9 showed 0.00006 and 0.0 g L^{-1} of nitrogen on day 10 of the culture and a high concentration of biopolymers. The growth conducted with inoculum adaptation showed 100% nitrogen consumption before 10 days of assays, and the yield of biopolymers exceeded that of the assays that were not previously adapted. Samantaray et al. (2011), in *Aulosira fertilíssima* grow observed that the production of phb is connected to a source of nitrogen limitation in the medium. When this nutrient is limited, PHB production is stimulated. According to Koller et al. (2008), in the stationary phase, when the nitrogen limitation occurs, carbon flux is applied in three directions: power maintenance; PHB production and storage of the carbon source; and excretion of the intermediary metabolites of the cells.

When grown under nitrogen limitation, PHB synthase enzyme was found in the membrane of the cyanobacterium *Synechocystis* PCC6803 (Hauf et al., 2013). The PHB synthase is activated by acetyl phosphate. The second enzyme involved, called phosphotransacetylase, converts acetyl CoA to acetyl phosphate. It is regulated by the acetyl CoA concentration and the ratio of carbon to nitrogen (C/N) in the cell. Thus, the acetyl phosphate can act as a sign of C/N balance that affects the PHB synthesis metabolism (Kessler and Witholt, 2001). According to Lee (1996), the production of polyhydroxyalkanoates is most effective when nutrients such as nitrogen and phosphorus are limited but still present in the medium. The premature absence of nutrients can cause cell death and consequently will not produce PHB.

The maximum carbon consumption in cultures without adaptation of inoculum was 21.7% in assay 4, which contained the highest concentration of sodium bicarbonate (8.4 g L^{-1}). Assays 3 and 7, which also had 8.4 g L^{-1} of $NaHCO_3$ in the medium, showed 19.0 and 17.2% carbon consumption, respectively. Only assay 8, grown with 8.4 g L^{-1} of sodium bicarbonate, had a lower uptake (6.5%), where the difference in this experiment was the nitrogen concentration (0.25 g L^{-1}) and P (0 5 g L^{-1}).

The nutrients added to the culture medium do not act alone on the growth and bioproduct formation of the microorganisms. This interaction of nutrients can cause a change in the concentrations of these components, which can direct the use of the same substrate differently and can stimulate an increase or reduction in use by the cells.

The assay in which adaptation of the inoculum was conducted showed a carbon consumption of 50.0% of the total added. The previous adaptation of the inoculum to the condition in which the microalgae will be exposed during growth causes the cell to be stable in this nutritional status. Thus, the microorganism does not suffer stress due to the medium change, and in addition, it already has the intracellular components needed to metabolize these active nutrients. The culture that had the same concentrations of carbon, nitrogen and phosphorus but that had not been inoculum-adapted showed a 17.2% carbon consumption.

The assays that had the largest concentrations of phosphorus (0.3 and 0.5 g L^{-1}) had the maximum consumption values (70.0 and 74.0%). Among the cultures without adaptation, assay 7 had the highest concentration of biopolymers (30.7%) and phosphorus consumption.

Sharma and Mallick (2005) kept *Nostoc muscorum* for 21 days in a medium that contained different carbon sources and later started growth with a phosphorus deficiency. The authors observed that the maximum yield of PHB was obtained at time zero, when the phosphorus concentration was higher. In cultures with *Synechocystis*, Wang et al. (2013) observed that the quantity of phosphorus reached undetectable values and thus cell growth did not reach the concentration that was required to promote the production of PHB. Thus, the authors added 30.0 mg L^{-1} of potassium phosphate to the assay, and after three days of culture, the concentration of PHB was significantly increased. These results indicate that the absence of phosphorus does not favor the production of biopolymers. Phosphorus is an important nutrient for the production of ATP. Excess ATP causes a reduction in oxidative phosphorylation and an accumulation of reduced coenzymes (NADH), which leads to the formation of PHB, whose metabolic pathway reoxidizes these coenzymes (Dawes and Sênior, 1973).

The assay with the inoculum adaptation showed less phosphorus consumption (42.6%) when compared to the assays without adaptation (74.0%). In cultures that were performed with *Synechocystis* sp. PCC 6803 and phosphorus limitation, there was an increase of 11.0% in the concentration of the biopolymers compared to assays with no phosphorus limitation.

The biopolymers extracted from *Spirulina* sp. LEB 18 had an initial degradation temperature of 163.5 to 208.8°C (Table 5). The commercial PHB presented the TG_0 of 233.4°C. Samantaray et al. (2011), showed the initial temperature of PHB degradation to 174.0°C for *Aulosira fertilissima*. The polymer degradability characteristics depend on the chemical nature of the raw

Table 5. Initial temperature degradation responses (TG_0, °C), final degradation temperature (TG_f, °C) and non-degraded material (IP, %) of the biopolymers extracted from *Spirulina* sp. 18 LEB.

Assay	TG_0	TG_f	IP
1	189.1	332.2	31.4
2	163.5	322.7	30.9
3	179.3	355.8	33.3
4	168.4	324.1	32.7
5	183.3	333.5	40.2
6	178.8	324.0	36.2
7	203.6	305.0	33.2
8	185.6	337.5	36.7
9	189.6	337.3	40.2
7a	208.8	316.4	36.7
Commercial PHB	233.4	293.9	2.3

material offered as a carbon source, the environmental conditions for the operation of the process and the selected microorganism. Thus, the polymer characteristics can be directed during the process (Castilho et al., 2009).

The final degradation temperatures varied from 305.0 to 337.3°C. The assay in which adaptation of the inoculum occurred showed TG_0 and TG_F values of 208.8 and 316.4°C, respectively. Assay 7 and the inoculum-adapted assay showed initial and final temperature values closest to the commercial PHB degradation compared to the other cultures performed.

The profiles of the DTG curves of the extracted biopolymer samples showed little reduction in weight before the start of the degradation of the polymer. This reduction is due to the moisture of the samples, and the same was not true in the commercial sample, which had appropriate treatment and packaging to not absorb moisture.

The commercial PHB sample showed a constant weight after the final degradation temperature, because this sample has only a small amount of non-degraded material (2.3%). However, the same behavior is not observed in samples of biopolymers that were extracted from microalgae. In this sample, the presence of non-degraded material causes the weight to continue decreasing with increasing temperature even after the end of the biopolymer degradation. This finding can be confirmed by the high content of the non-degraded material contained in the samples, which reached 40.2% in assay 9.

Conclusions

In the growth of *Spirulina* sp. LEB-18 at different nutrient concentrations, it has been found that the microalgal growth was inversely related to the synthesis of the biopolymers. The maximum cell growth occurred in assay 6, with a concentration of 0.62 g L^{-1}, and the biopolymer yield was 13.4%. While in assay 7, the cell growth was 0.5 g L^{-1}, and the biopolymer accumulation was 30.7%. By making the reproducibility of the best conditions with prior adaptation to inoculation, an increase in the biopolymer yield of 20.5% was observed. Through the assays, the importance of the carbon source in the production of biopolymer was observed, with a maximum synthesis observed in the assay that had the highest consumption of carbon compared to the others.

Biopolymer production is more efficient when nutrients such as nitrogen and phosphorus are restricted but present in the medium. The initial and final degradation temperatures of the assays that had the maximum biopolymer yield were close to the value of commercial PHB (233.4 to 293.9°C). Biopolymers, especially polyhydroxyalkanoates, play an important role in the plastics market due to their biodegradability and the use of renewable resources in their production, which reduces environmental problems such as the pollution caused by plastics of petrochemical origin.

Conflict of interest

Author have not declared any conflict of interest.

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A simple nested polymerase chain reaction for differential identification of *usp*I and *usp*II genes encoding uropathogenic specific protein of uropathogenic *Escherichia coli*

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The gene encoding a protein which was designated as uropathogenic specific protein was identified on pathogenicity island of uropathogenic *Escherichia coli*. The *usp* gene is mainly classified into two types (*usp*I *and usp*II) depending on the difference in DNA sequence at the 3' region. A simple nested polymerase chain reaction assay was applied to differentiate *usp*I and *usp*II. The results indicate the presence of 22 *usp*I and 42 *usp*II genes positive isolates. This study will be useful for accuracy of uropathogenic specific protein pathogenicity island subtyping method for epidemiological study of uropathogenic *Escherichia coli*.

Key words: Uropathogenic *Escherichia coli*, uropathogenic specific protein, nested polymerase chain reaction, gene typing.

INTRODUCTION

A 4167-bp putative pathogenicity island (PAI) commonly associated with Uropathogenic *Escherichia coli* (UPEC) strains was identified while searching Zot (zonula occludens toxin) – like genes in these strains (Kurazono et al., 2000). Using molecular methods, a gene encoding a protein which was designated as uropathogenic specific protein (USP) was found on PAI. The *usp* gene is followed by three small open reading frame units (*orfUs*): *orfU1*, *orfU2* and *orfU3* of 98, 97 and 96 amino acids, respectively (Kurazono et al., 2000; Nakano et al., 2001).

The precise mechanism of USP on the urinary tract was not clear. In one report, it was pointed out that the USP protein and small OrfUs following this protein showed high homology to the S-type pyocins produced

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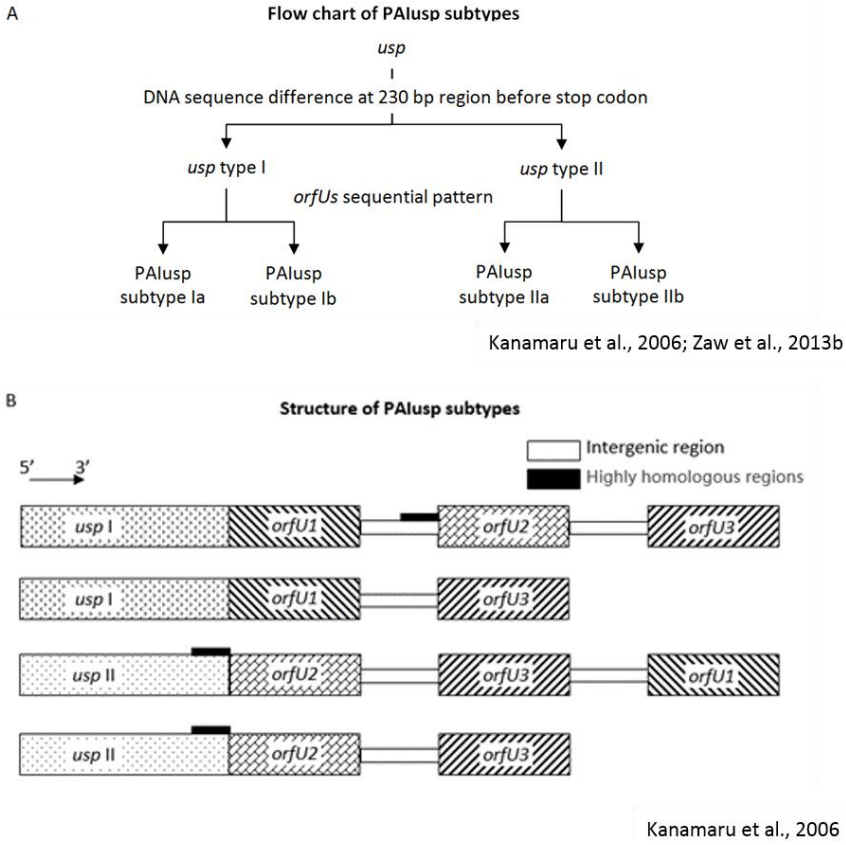

Figure 1. A. Flowchart of PAIusp subtypes classification**.** The flowchart was drawn for this study. The information for this flow chart was taken from references mentioned under the figure. **B.** Diagram showing structures of genes in each subtype of PAIusp. The black bars indicate the location of the highly homologous regions of 3' half of inter-genic region between *orfU1* and *orfU2* following *usp*I and 3' diverse region of *usp*IIs. This information was obtained from the alignment of DNA sequences from NCBI database gene bank accession no. AB056434 and AB056437 (Nakano et al, 2001).

by *Pseudomonas aeruginosa* (*P. aeruginosa*) strains and its immunity proteins (Parret and De Mot, 2002). Nuclease colicins need the specific immunity (inhibitor) proteins which make the colicins-producing bacteria to avoid committing suicide (Papadakos et al., 2012). These two literatures pointed out that USP is bacteriocin like protein and the OrfUs were immunity protein to protect *Escherichia coli (E. coli)* from the effect of USP. In one study, it was demonstrated that USP together with OrfU2 was applied to mammalian cells and genotoxic activity was observed (Nipič et al., 2013). OrfU3 (immunity protein – 3) had DNA and RNA binding activity and prevents suicidal effect of genotoxin USP on *E. coli* (Črnigoj et al., 2014). In the recent study, it was found that USP was purified after co-expression of *usp* gene and *orfU1* and characterization of USP was done. In the same study, like other bacteriocins, USP was observed to have non-specific DNase activity (Zaw et al., 2013).

Virulence genes are the molecular markers for epidemiological studies. For example *fimH* gene, *pap* gene and *usp* gene are commonly used for molecular epidemiology of UPEC (Dias et al., 2010; Bauer et al., 2002; Karimian et al., 2012). In the very recent study, the profiling of virulence genes like *tox*A, aprA, *rhl*AB, *plc*H, *las*B and *fli*C of *P. aeruginosa* isolated from patients with urinary tract infections (UTI) was done. The results showed variable distribution of virulence genes. The study highlights the virulence genes are useful diagnostic markers for clinical *P. aeruginosa* strains isolated from UTI (Sabharwal et al., 2014).

The reasons that *usp* gene encoding USP protein was predominant in UTI isolates, it was classified into two types due to the difference in DNA sequence and the sequential position of associated three *orfU*s immediately downstream of *usp* gene was not the same (that is orfU1*, orfU2 and orfU3* are in sequence, or *orfU2, orfU3 and*

Table 1. Nucleotide sequences of primers used in this study.

Name of primer	Primer sequences	Size of PCR products
Nest 1 primers	N1F:5'- GCT TTC ATC AGG AAC TCG CTG G –3' N1R:5'-TTA TCT CCT GTA GAA TTT CAT CAT G -3'	380 bp (this study)
*usp*I primers	MYF:5'-ATT CCC CCT ATG TCC CTG AG -3' MYR: 5'-TCC ACC ACC ATG TTC TAT A-3'	101 bp (this study)
*usp*II primers	MY2F: 5'-CAG GAT CCG GTG TTG ATA-3' MY2R: 5'-GCT GCC ACC ATA TTC AAC T-3'	161 bp (this study)

orfU1 are in sequence, or sometimes only two *orfU*s are present with different combination depending on isolates) make *usp* gene possible to be useful epidemiological marker. PAIusp subtyping method, which characterizes UPEC isolates from the molecular level, depends on (1) *usp* gene typing and (2) arrangement of 3 *orfU*s following *usp* gene and PAIusp subtypes are Ia, Ib, IIa and IIb (Figure 1A and B) (Kanamaru et al., 2006). However, researchers up to now relied on *orfU*s sequential order for PAIusp subtyping and this fundamental method of *usp* typing was not mentioned in their procedure. Therefore, simple nested PCR assay was introduced in this study with nest duplex PCR using newly designed primer sets for two *usp* genes to differentiate *usp*I and *usp*II.

MATERIALS AND METHODS

Ethical clearance was applied to University ethical committee and National Medical Research Registration (NMRR) and approval was already obtained. Sixty-four UPEC isolates positive for *usp* gene stocked in microbiology laboratory, Faculty of Medicine and Health Sciences, Universiti Malaysia Sabah were included in this study. These UPEC isolates were derived from urine samples sent to microbiology laboratories of Hospital Queen Elizabeth and Papar Hospital for culture and sensitivity during January to March, 2013. The two hospitals were located around Kota Kinabalu, Sabah, Malaysia. We have checked the urine samples for significant bacteriuria. Positive controls were the strains which were shown to have *usp* I and *usp* II genes by DNA sequencing and negative control was *E. coli* ATCC 25922.

DNA sequence of nest 1 forward and reverse primers and newly designed forward and reverse primers for *usp*I and *usp*II genes are shown in Table 1 with their sizes of PCR products expected. The primer designation was performed following the guidelines by Burpo (2001).

The bacterial isolates were inoculated in 3 ml of Luria-Bertani broth and incubated at 37°C for 18 h. The bacterial DNA was extracted by boiling method (Abdallah et al., 2011; Ifeanyi et al., 2015). In case of primary PCR, 5 µl of DNA as template was added to PCR reaction mixture (25 µl) containing 10 µm each primer, dNTPs 10 mmol, 1x buffer and 1 unit of Taq polymerase (Takara). PCR was done in Applied Biosystems Thermocycler with PCR conditions as initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 20 s, extension at 72°C for 30 s. The size of PCR products was checked by florosafe in a 1.5% agarose gel, and recorded by gel documentation apparatus Alpha Imager® HP System. For nest PCR, which was duplex PCR, the PCR ingredients were same as above mentioned procedure with the exception of 0.1 µl nest 1 product was used as the template and two primer sets were added. PCR conditions were initial denaturation at 94°C for 5 min, 15 cycles of denaturation at 94°C for 10 s annealing at 55°C for 10 s, extension at 72°C for 20 s and 2.5% agarose gel was used to check PCR products. It took less than 2 h after conditions were standardized for the whole nested PCR.

RESULTS AND DISCUSSION

In the previous studies on USP, the epidemiologically important PAIusp subtyping method was used to characterize the UPEC isolates. There is absence of gene typing method which is more fundamental step when compared with the sequential order of *orfUs* in their study (Kanamaru et al., 2006). In this study, the set of primers which could amplify the region where *usp*I *and usp*II have heterologous sequences were designed for the primary PCR. For the nest PCR, the two primer sets, each having the most heterologous sequences in *usp*I and *usp*II genes in the 3' regions were used for duplex PCR which could differentiate two genes. PCR products sizes were convenient for the gel electrophoresis by 1.5% and 2.5% agarose gel, which were 380 bp for the primary PCR and 161 bp and 101 bp for the nest PCR respectively. All the 64 samples were positive in the primary PCR. This primary PCR amplify the regions which include where the two genes are heterologous. This PCR has advantage in leaving the intergenic region which will disturb the following nest PCR and this information will be explained in the discussion. In the nest PCR, 22 samples were positive for *usp*I gene and 42 were positive for *usp*II gene. The primer pairs have succeeded in differentiation of two genes. The 7 UPEC positive isolates for *usp* Nested PCR were shown in Figure 2A and B.

Up to now, there were two reports about the nature and action of USP. One study proved that it has the non-specific DNase activity (Zaw et al., 2013). It was observed in the other study that it has genotoxic effect when applied to mammalian cells (Nipič et al., 2013).

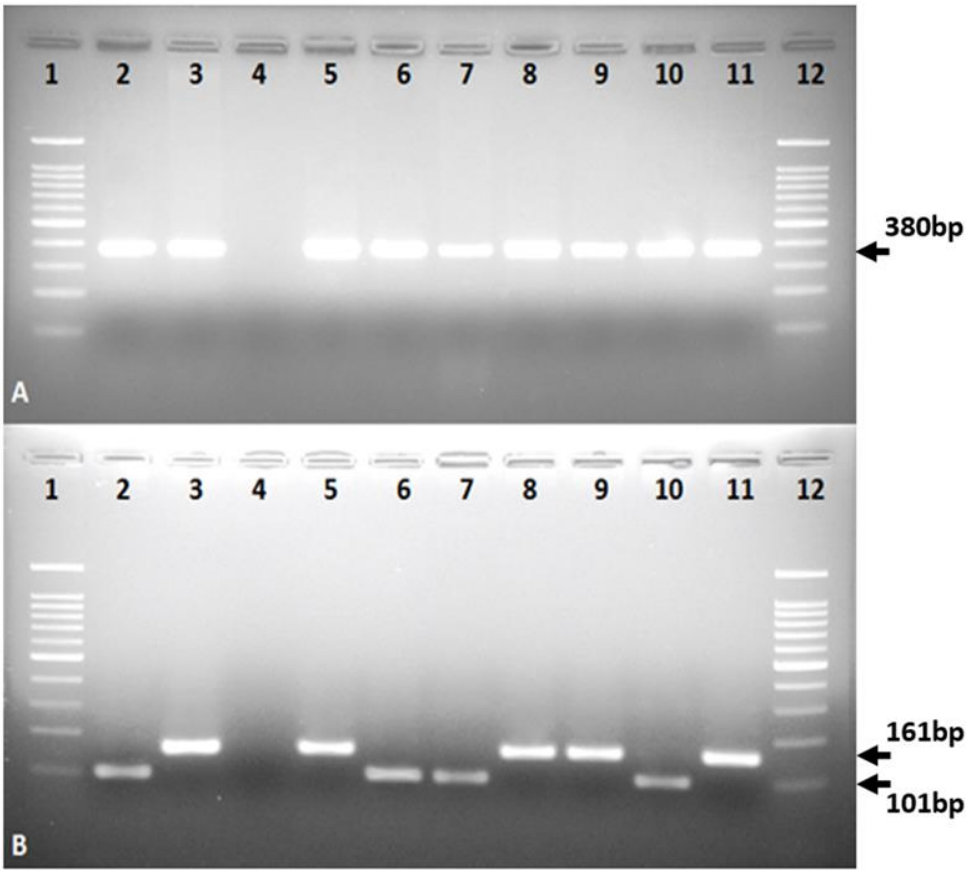

Figure 2. Nested PCR assay for differentiation of *usp*I and *usp*II . A. Gel electrophoresis picture of primary PCR product of UPEC isolates containing *usp* gene. Lane 2 and 3 are isolates for positive control *usp*I, *usp*II respectively. Lane 4 is an isolate used as negative control lacking of *usp* gene. Lane 5 to 11 were isolates investigated in this study all giving rise to 380 bp PCR products. Lane 1 and 12 were 100bp molecular markers. B. Gel electrophoresis picture of Nest PCR product having *usp*I or *usp*II. Lane 2 and 3 are positive control *usp*I, *usp*II showing 101 bp and 161 bp respectively. Lane 4 is negative control. Lane 6, 7 and 10 are PCR products *usp*I with the 101 bp DNA fragment size and lane 5, 8, 9 and 11 are PCR products *usp*II gene having 161 bp in size. Lane 1 and 12 were 100 bp molecular markers.

Although the action of USP was concluded by different researchers according to their findings, the value of *usp* gene and its *orfUs* following this gene is classification of UTI isolates into four PAIusp subtypes which are of epidemiologic significance.

Structural and diversity of *usp* genes indicated that *usp*I and *usp*II were heterologous at 3' end in the previous study (Nakano et al., 2001). This diversity occurred at 3' 230 bp region upstream of stop codon. Because of this diversity and sequential orientation of *orfU*s, epidemiologically important PAIusp subtypes were well known. PAIusp subtype IIa was highly prevalent in the studies in Japan (Kanamaru et al., 2006). However, PAIusp subtyping relied on sequential orientation of *orfU*s 1, 2 and 3 in that study. It is necessary to lay down the method for *usp* gene typing. If PAIusp subtyping pattern becomes different from currently classified four types in new isolates, *usp* gene typing will be helpful in characterization of UPEC isolates.

PCR-RFLP and direct duplex PCR were tried for *usp* gene typing. Although these are part of this study, these were not mentioned in this report. However, the first method depends on *Hpa* II restriction site and if there are single nucleotide polymorphisms (SNPs), typing can be mistaken. In case of duplex PCR, the *usp*II gene is highly homologous to 3'intergenic region between *orfU1* and *orfU2* of *usp*I gene (black bars in Figure 1B) giving rise to two reactive bands giving wrong impression of having two gene types in the detection of some *usp*I isolates. In this nested PCR assay, we can leave the intergenic region in primary PCR so that nest PCR can provide the correct gene type. We succeeded in the gene typing method for

*usp*I *and usp*II using simple and rapid nested PCR assay by taking advantage of principle of nested PCR.

This is the first study for gene typing of *usp*I and *usp*II, which is the basic step in epidemiologically important PAIusp subtyping. In the previous work, PAIusp subtyping was done depending on *orfUs* sequential patterns without gene typing. Gene typing of *usp*I *and usp*II is difficult because of the abovementioned reasons. However, nested PCR is valuable for overcoming these obstacles so that this study has contributed to the accurate method of PAIusp subtyping if there is extra subtype beyond the currently existing four subtypes.

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Conflict of interest

The authors have no conflict of interest to disclose.

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Full Length Research Paper

Expression of peptide nanoparticles containing a porcine reproductive and respiratory syndrome (PRRS) virus epitope in plants

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Porcine reproductive and respiratory syndrome is one of the most devastating diseases affecting the pig industry. The licensed vaccines available present several shortcomings and consequently many groups around the world are actively working towards developing an efficacious vaccine. In this work, we have fused the epitope B of the GP5 protein from the PRRRS virus to peptide nanoparticles and expressed the construct in plants in a transient manner. It was shown by transmission electron microscopy that the chimeric protein nanoparticles can be efficiently synthesized and self-assembled inside plant cells. By real-time polymerase chain reaction (PCR), it was also demonstrated that the chimeric constructs are efficiently transcribed. There exists a high potential for these nanoparticles to serve as platforms for vaccines. In the next phase of the project, we will immunize mice to show immunogenicity and pigs, which will be later challenged with a circulating strain of the virus.

Key words: Peptide nanoparticles, epitopes, porcine reproductive and respiratory syndrome virus (PRRSV), GP5, vaccines.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is one of the most important viral diseases affecting the global pork industry, with annual losses of billions of dollars per year worldwide (Neumann et al., 2005; Holtkamp et al., 2013; Zhang et al., 2014). The syndrome is characterized by reproductive and respiratory failure and it is associated with abortions and infertility, respiratory distress in nursing pigs, poor growth and increased mortality (Rowland and Morrison, 2012; Hu and Zhang, 2014). It is caused by PRRS virus (PRRSV), which is a member of the *Arteriviridae* family in the *Nidovirales* order (Lunney et al., 2010; Snijder et al., 2013). The genome of PRRSV, a single-stranded positive-sense RNA of about 15 Kb in length with a methyl capped 5' and a 3'-polyadenylated tail, expresses a wide range of accessory and structural proteins via two distinct transcription mechanisms (Han and Yoo, 2014; Kappes and Faaberg, 2015). The PRRSV genome is

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polycistronic and originates 10 overlapping open reading frames (ORFs) (ORF 1a, 1b, 2, 2b, 3-7) (Kappes and Faaberg, 2015). ORFs 2-5 encode glycosylated membrane proteins GP2a, GP3–GP5 plus GP5a. GP2, GP3 and GP4 form a trimeric complex resulting in the minor glycoprotein complex which enhances viral entry and is heavily N-glycosylated (Kappes and Faaberg, 2015). The nucleocapsid protein (N) is encoded by ORF7. N is the major structural element within the PRRSV virion and forms disulfide-linked homodimers; It functions to package the viral genomic RNA and is the only known structural protein, which does not encode a transmembrane domain (Dokland, 2010).

The original work suggested that only GP5, GP6 (M) and N made up the majority of the protein content of PRRSV (Mardassi et al., 1996; Dokland, 2010). Recently, the nsp2 protein, which is coded for by the most variable region of the genome, was demonstrated to be incorporated into virions of several PRRSV strains as a set of differently sized protein isomers (Kappes et al., 2013, 2015b). This unexpected result increases the number of viral proteins to at least 10 (full-length nsp2 and its isomers, nsp2TF, GPs2–5, E, M, N, ORF5a), that are exposed to the porcine immune system on entry of PRRSV into swine alveolar macrophages (Fang et al., 2012; Veit et al., 2014). The enormous genetic and protein variation of all of these structural proteins, from the least conserved nsp2 to the most conserved M protein shows the complexity and plasticity of the PRRSV genome and virion structure (Rascón-Castelo et al., 2015; Kappes and Faaberg, 2015a).

GP5 is a glycosylated protein of approximately 25 kDa and although it carries the major neutralizing epitope (epitope B) (Mardassi et al., 1996; Ostrowski et al., 2002; Plagemann et al., 2002), it is also the most variable viral protein (Dokland, 2010; Kappes et al., 2015a).

PRRSV mutates at an extremely rapid rate, even for a RNA virus (order of 10^{-2} changes/amino acid site/year, as compared to 10^{-3} to 10^{-5} for HIV or hepatitis C virus) than that seen in other RNA viruses (Hanada et al., 2005) and this is probably due to more rapid replication, rather than to a higher error rate (Murtaugh et al., 2010).

Control of PRRSV is primarily focused on prophylactic vaccines. The vaccines currently licensed consist of modified-live and killed-virus, but live PRRS vaccines may revert to virulent virus under farm conditions (Botner et al., 1997) and the efficacy of both vaccines may vary upon heterologous challenge (Diaz et al., 2006; Cano et al., 2007; Rebeaud and Bachmann, 2012). None of the available vaccines is able to prevent respiratory infection or pig-to-pig transmission (Kimman et al., 2009).

The unsatisfactory efficacy and safety of current PRRSV vaccines has driven the continuous efforts for developing better and safer vaccines. However, development of new PRRSV vaccines faces many challenges because of the high variability and the diverse mechanisms that the virus employs to evade the immune response (Huang et al., 2015; Salguero et al., 2015). A number of strategies including recombinant production of different PRRSV antigens in various systems have been tested (Qiu et al., 2005; Jiang et al., 2006; Zheng et al., 2007; Li et al., 2009a, b; Cruz et al., 2010; Chen and Liu, 2011; Chia et al., 2011; Vimolmangkang et al., 2012; Chan et al., 2013; Nam et al., 2013).

The epitope B of GP5 protein has been suggested as a promising vaccine candidate (Wissink et al., 2003; Snijder et al., 2013). It is well known that small, single peptides are poorly immunogenic and therefore their use as individual peptides is not recommended (Bae et al., 2009). However, they could be presented to the immune system on the surface of nanoparticles, which are highly repetitive. These repetitive patterns are efficiently recognized by the mammal immune system. Epitopes that are capable of inducing an immune response can be displayed repetitively at high density on different types of nanoparticles (Pimentel et al., 2009). The key role of epitope density was illustrated in a study showing that nanoparticles decorated with a peptide at different density produce high IgG responses only at the highest density (Jegerlehner et al., 2002) and that only highly repetitive antigens could break B cell unresponsiveness and induce self-specific antibody responses (Bachmann et al., 1993; Chackerian et al., 2002).

We decided to employ peptide nanoparticles as a vehicle of presentation of the GP5 epitope B of PRRSV to the immune system. The nanoparticles are formed by pentameric and trimeric motifs derived respectively from coiled coil domains of a monomer taken from the cartilage oligomeric matrix protein and a *de novo* designed oligomer (Babapoor et al., 2011; Raman et al., 2006). These pentameric and trimeric coiled coils oligomers, use the threefold and fivefold symmetry of an icosahedron to yield a self-assembling nanoparticle (Raman et al., 2006). Self-assembly into a predicted regular icosahedral nanoparticle of about 16 nm of diameter and 473 KDa in size occurs when the coiled-coil domains of different monomers associate to form the icosahedral nanoparticle. A nanoparticle with this type of architecture can be used as a vaccine by extending the ends of the monomer with an epitope sequence (Babapoor et al., 2011). Nanoparticles of this type have only been expressed in bacterial systems and employed to express epitopes of *Plasmodium berghei* (Kaba et al., 2009), avian influenza (Babapoor et al., 2011) and severe acute respiratory syndrome virus (Pimentel et al., 2009).

In this work, as a first step towards using the peptide nanoparticles decorated with the GP5 epitope B of PRRSV as a candidate vaccine, our aim was to demonstrate that peptide nanoparticles, expressing the epitope B of GP5, were able to assemble adequately in a plant expression system. Why use plants? Over the last 10 years, the use of plants as platform has won relevancy because of its many advantages (Kumar et al., 2013). Plants have become a very attractive system for

Table 1. Peptide nanoparticle sequences.

Construction	Sequence
M-GP5	MGHHHHHHASWKWDGGLVPRGSDEMLRELQETNAALQDVRELLRQQVKQITFLRALLMGGRLLARLEELERRLEELERLEELERAINTVDLELAALRRRLEELARGGSGSHLQLIYNL
T-GP5	MGHHHHHHASLVPRGSHLQLIYNLSSGSLYRLTVIIDDRYESLKNLITLRADRLEMIINDNVSTLRALLMGGRLLARLEELERRLEELERRLEELERAINTVDLELAALRRRLEELAR

Pentameric coiled coil domain (green), trimeric de novo designed coiled coil domain (blue), tetrameric coiled coil (pink) and PRRS GP5 Epitope B in orange. Linkers other amino acid residues and his-tags are shown in black (Babapoor et al., 2011).

expression of many antigens and the recent approval of the first plant-made biologics by the U.S. Food and Drug Administration (FDA) for plant production and commercialization has opened the door for more products to be developed (Chen and Lai, 2013).

MATERIALS AND METHODS

Constructions

The peptide nanoparticles M-GP5 and T-GP5 sequences were synthesized by GenScript from the nanoparticles sequences previously reported by Babapoor et al. (2011). These nanoparticles will display the antigen of interest in a repetitive manner in high density in both monomeric (M-GP5) or tetrameric form (T-GP5) which are different conformations of the structure (Babapoor et al., 2011).

The conserved epitope B (SHLQLIYNL) of GP5 PRRSv protein (Ostrowski et al., 2002) was genetically fused to the nanoparticle sequence (Table 1). *E. coli* DH5α cells were transformed with M-GP5 or T-GP5 and incubated overnight at 37°C on LB medium plates in the presence of carbeniciline (50 mg/L). DNA plasmid was extracted from a single colony. The 365 or 361 pb fragments corresponding to M-GP5 or T-GP5 were digested with BsaI and subcloned in the BsaI site of pICH31070 to obtain pICH31070-M-GP5 or pICH31070-T-GP5. Vectors pICH15879, pICH14011, pICH-GFP, pICH31070-M-GP5 and pICH31070-T-GP5 were then transformed in *Agrobacterium tumefaciems* GV3101. Expression vectors were all kindly provided by Dr. Yuri Gleba (Icon Genetics).

Agroinfiltration

Infiltrations were done as described by our laboratory before (Coconi-Linares et al., 2013) Briefly, the GV3101 cells were incubated in YEB medium at 28°C until the OD_{600}nm = 1.5. Then acetosyringone (200 µM) was added and the cultures were incubated at room temperature for two hours more. Bacteria were harvested by centrifugation at 3000 xg and the pellet of each culture was resuspended in agroinfiltration buffer (10 mM MES pH 5.5, 10 mM MgSO4) to a final OD_{600}nm = 0.15. Equal volumes of each culture were mixed and the bacteria suspension used for agroinfiltration. Leaves of six to seven weeks-old *Nicotiana silvestris* plants grown in a controlled ambient chamber were infiltrated with bacterial suspension using a syringe without needle. Infiltrated plants were incubated at 25°C (16/8 ligth/darkness) for 6-10 days.

Extraction of total RNA and cDNA preparation

Total RNA was extracted from 100-200 mg of frozen infiltrated leaves with the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. All RNA samples were analyzed by agarose gel electrophoresis and visual inspection of the ribosomal RNA bands upon ethidium bromide staining. Total RNA samples (5 µg) were reverse-transcribed to generate single-stranded cDNA using an oligo dT18 primer and 200 units of SuperScript III reverse transcriptase as described by the manufacturer (Invitrogen, Carlsbad, CA, USA).

Table 2. Sequence of primers for quantitative real time PCR.

Primer	Forward (5'-3')	Reverse (5'-3')
M-GP5	agaaacaaacgcagcactacag	gccagtaatcttcctcccataag
T-GP5	accgacttactgtgataattgacg	tcgtgccaataatcttcctccc
L23	aaggatgccgtgaagaagatgt	gcatcgtagtcaggagtcaacc
PP2A	gaccctgatgttgatgttcgct	gagggatttgaagagagatttc

Gene expression analysis by quantitative real-time PCR (qRT-PCR)

The single-stranded cDNA was diluted five-fold in sterile milli-Q water prior to qRT-PCR. Amplifications were performed using SYBR Green and run in triplicate in 96-well reaction plates with the real-time PCR System CFX96 (BioRad Laboratories Incorporated, USA). Amplification reactions were prepared in a total volume of 20 µl as indicated by the manufacturer: 2 µL of cDNA, 2 µL of each amplification primer (2 µM), 10 µL of iQ SYBR® Green Supermix (BioRad Laboratories Incorporated Hercules, CA) and 4 µL of sterile milli-Q water. qRT-PCR was performed in triplicate for each sample. The primers for the endogenous genes used as reference for normalization were designed based on expression stability data in *Nicotiana tabacum* and *Nicotiana benthamiana* previously reported (Liu et al., 2012; Schmidt and Delaney, 2010). The primers for amplification of M-GP5 and T-GP5 were design from the sequence of each peptide nanoparticle sequence (Table 2). Primer design was performed using Primer-Blast (Ye et al., 2012), and Oligo Evaluator™ software (Sigma-Aldrich). The relative expression was determined by evaluating the expression by the 2–ΔΔCT method (Livak and Schmittgen, 2001).

Total soluble protein extraction and SDS-PAGE

Infiltrated leaves were cut and immediately frozen in liquid nitrogen. Frozen tissue was macerated with mortar and pestle and mixed with extraction buffer (50 mM sodium phosphate pH 7.4, 100 mM NaCl, 10 mM EDTA pH 8.0, 5 mg/mL ascorbic acid, 1mM PMSF, inhibitor cocktail, 0.1% triton X-100, 1% sodium bisulphite) using 1

mL of extraction buffer for each gram of fresh tissue. The mix was incubated for 10 min at room temperature and then centrifuged at 14,000 x *g* for 20 min at 4°C. The supernatant was filtered with three layers of miracloth and then with 0.8, 0.45 and 0.22 µm cellulose acetate filters. Protein quantification in total soluble extracts was estimated by the Bradford (1976) assay with bovine serum albumin as a protein standard. 30 µg of total soluble protein were separated by 12% SDS-PAGE. The samples were mixed with 5X loading buffer with β mercaptoethanol (Sigma) and then were boiled for 5 min at 95°C. After migration, the gels were fixed in water containing 10% (v/v) acetic acid and 40% (v/v) methanol, and then washed three times in water. The proteins were visualized by staining with the colloidal Coomassie blue (Sigma).

Peptide nanoparticles purification

The purification protocol employed was based on those used by Hu et al. (2008) and Rao et al. (1994). Briefly, total soluble extracts were mixed with an equal volume of chloroform, stirred for 60 min at 25°C and then centrifuged at 12,000 xg for 20 minu at 4°C. The supernatant was recovered, loaded on a 10% sucrose cushion and centrifuged at 130,000 xg for 2.5 h at 4°C. The pellet was recovered and mixed in resuspension buffer (0.05 M sodium acetate, 0.008 M magnesium acetate, pH 4.5) and then separated on a 10-40% sucrose gradient at 130,000 xg for 1 h at 4°C. Bottom fraction was collected and centrifuged again at 245,400 xg for 2 h at 4°C. Peptide nanoparticles were then purified with PrepEase® Histidine Tagged protein purificaion kit (USB Corporation, Cleveland Ohio USA).

Electron microscopy

Fresh samples were adsorbed on formvar/carbon-coated cupper grids and negative stained with 2% phosphotungstic acid, pH 5.5 for 10 min. The stained peptide nanoparticles were observed by transmission electron microscopy (Morgagni series 5005, Phillips).

RESULTS

In this work, we aimed to confirm whether the peptide nanoparticles containing the GP5 epitope B of PRRSV could be obtained using the plant transient expression platform. To this end, two chimeric constructs were prepared and transiently expressed in leaves of *N. sylvestris*. The sequences had previously been codon-optimized for expression in plants. We designed two constructs, M-GP5 which yields icosahedral nano-particles whereas T-GP5 yields octahedral nanoparticles. According to Babapoor et al. (2011), M-GP5 seems to contain the highest density of the epitope. We will be testing them separately in future immunological challenges in mice and pigs. As a first step towards determining whether nanoparticles could assemble in plants, we purified the particles and performed an analysis by TEM. As shown in Figure 1, we were able to obtain particles in both cases with sizes between 16-25 nm. The size and morphology of the nanoparticles closely resembled those obtained in bacteria (Babapoor et al., 2011; Kaba et al., 2009*;* Pimentel et al., 2009*).* This data demonstrated that nanoparticles were successfully formed in the plant cytoplasm.

In order to confirm the expression of the particles in plants, we obtained the total soluble protein of plant extracts and separated them by SDS-PAGE. We observed that a protein with size about 15 KDa, corresponding to the monomer size of peptide nanoparticles in both versions, was clearly detectable in samples expressing the constructs M-GP5 or T- GP5 and it was not detectable in extracts of non-infiltrated plants (Figure 2). This band was similar in relative molecular mass to the purified protein.

To quantify the relative expression of the chimeric particles M-GP5 and T-GP5, we employed quantitative real time PCR using RNA from infiltrated and non-infiltrated plants. We observed that both constructs were detectable by this technique (Figure 3). Expression levels for infiltrated and non-infiltrated plants were normalized using values of the 18S (18S ribosomal RNA) and eEF1a (eucaryotic elongation factor 1α) constitutive, endogenous genes.

DISCUSSION

Many attempts have been made at developing an efficacious vaccine against PRRS. Current vaccines present several shortcomings but the main one is that the efficacy may vary upon heterologous challenge. This may be due to the high variability and the evasion mechanisms of the virus. Even though a number of viral antigens have been expressed in various systems, including plants, the efficacy of such an approach is still in doubt.

In this sense, it has been suggested that the mammalian immune system has evolved to detect repetitive patterns present in pathogens but absent in mammal, pathogen-associated molecular patterns (PAMP) (Bachmann and Jennings, 2010). The plant-based nanoparticles obtained in this work resemble those obtained by Raman et al. (2006) in *E. coli* in size and appearance and present a repetitive pattern.

When the sequence of these nanoparticles was expressed in *E. coli* and purified, no particles were obtained, they had to be subjected to several folding treatments *in vitro* for the particles to assemble (Raman et al., 2006; Pimentel et al. 2009). However, in our case, the nanoparticles were able to self-assemble spon-taneously in the plant cytoplasm without any further treatment. In this case, our platform might be superior to *E. coli* for nanoparticles expression and folding.

We were interested to determine if the protein making up the particles was detectable by SDS-PAGE. A band of about 15 kDa, which is the expected size of the monomer, was detectable in crude extracts from infiltrated plants but not in crude extracts from non-infiltrated plants. The fact that the unpurified protein was easily detectable in crude extracts by SDS-PAGE gels means that it is been synthesized at significant levels

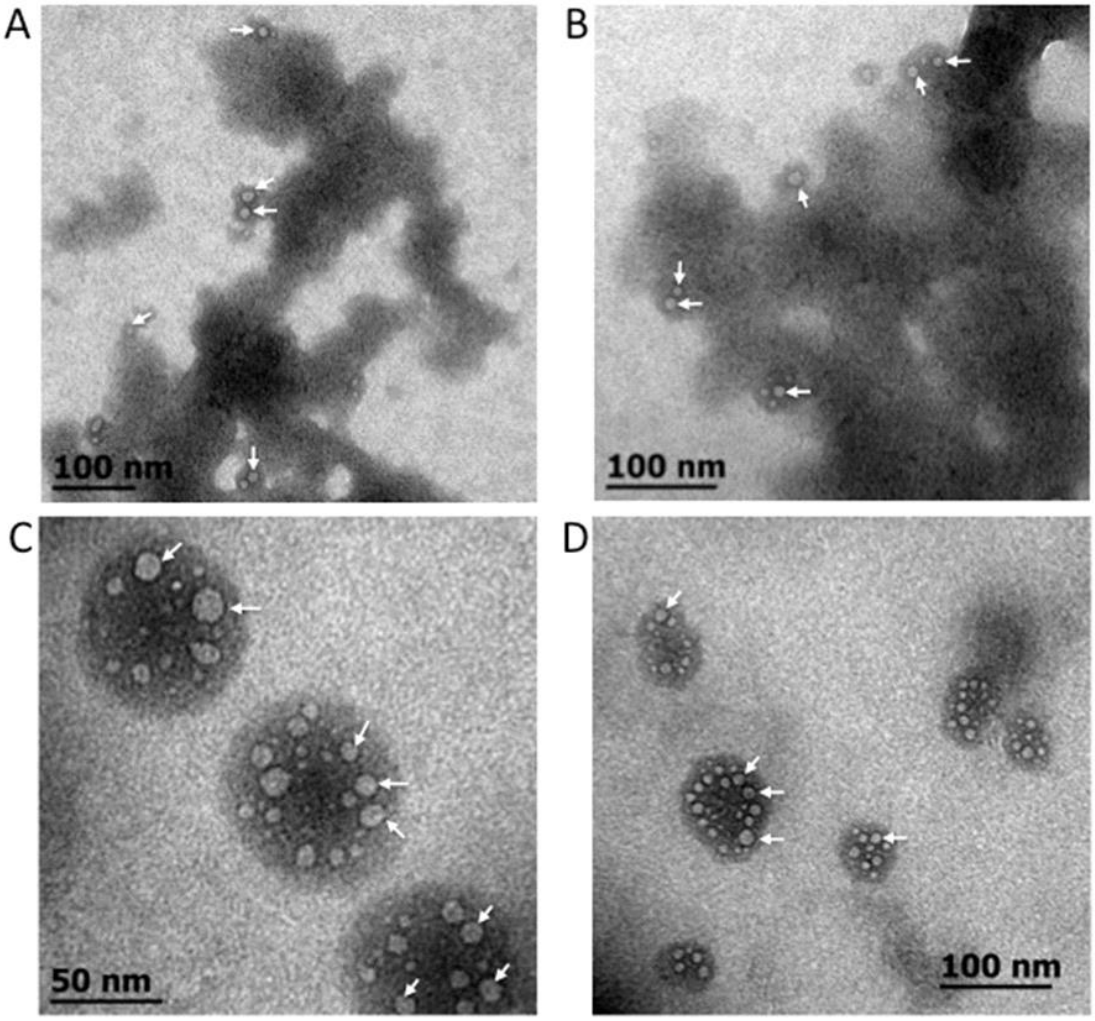

Figure 1. Electron micrographs of the peptide nanoparticles produced in plants. The particles were purified as described in the text and observed by TEM. A-B correspond to M-GP5 and C-D correspond to T-GP5. Particles were stained with 2% phosphotungstic acid at pH 5.5. The white arrows indicate 15-25 nm particles.

because it is known that only abundant proteins are detected by this technique (Virgen-Ortiz et al., 2013). We are in the process of obtaining specific antibodies against the nanoparticle protein and in the next step of the project, we will quantify the nanoparticle protein by ELISA.

In order to confirm transcription of the transgenes we employed qRT-PCR. Interestingly, T-GP5 seemed to express at a much higher level than M-GP5. This result indicates that both transgenes are being expressed in the plant cells but we have no explanation why one of them expresses much better that the other one.

In this work, we have shown that protein nanoparticles containing the epitope B from PRRSV can be synthesized and efficiently self-assembled inside plant cells. The potential for these nanoparticles to serve as platforms for vaccines is quite obvious. Recombinant antigens can be prepared in a matter of weeks to new variants of the antigen which makes this approach a powerful tool for a fast and effective response when new virus strains arise. Nevertheless, the platform has some limitations such as plant glycosylation, which is different from that of animal cells and the fact that antigen purification from plant cells is still a somewhat cumbersome process. In the next phase of the project, we will immunize mice to show immunogenicity with pigs, which will be later challenged with a circulating strain of the virus.

Conclusion

Peptide nanoparticles containing a PRRSV epitope can be efficiently expressed and self-assembled in plant cells.

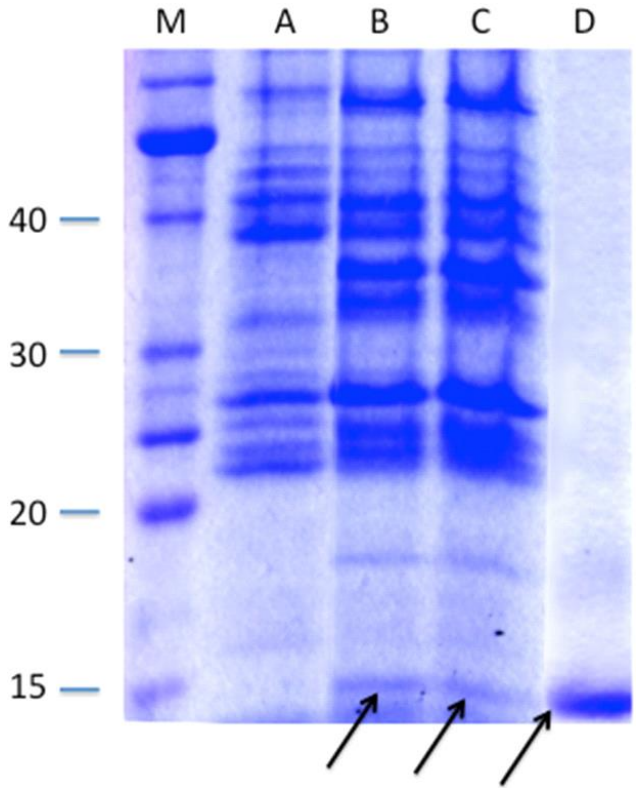

Figure 2. Analysis of total soluble proteins extracted from infiltrated and non-infiltrated plant by SDS-PAGE. Thirty micrograms of total protein were loaded per lane. Lanes are as follows: M, Molecular weight markers; A, non-infiltrated plants; B, plants infiltrated with M-GP5; C, plants infiltrated with T-GP5; D, purified protein from M-GP5. Arrows indicate the protein corresponding to the nanoparticles. Numbers on the left indicate the molecular weight markers in kDa.

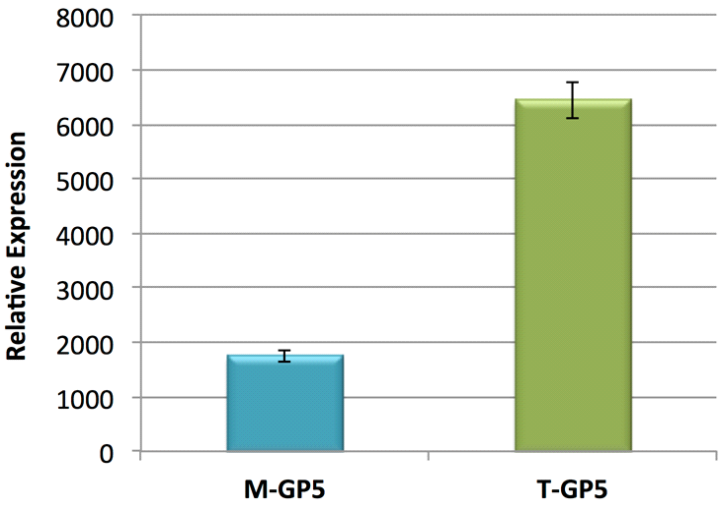

Figure 3. Analysis of the relative expression of M-GP5 and T-GP5 peptide nanoparticles by qRT-PCR. The relative expression of each gene (expressed as fold over the reference genes) was evaluated employing specific primers as described in the text. The expression of peptide nanoparticles was normalized against the expression of the endogenous, reference genes PP2 and L23.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Isolation and identification of tick borne bacterial pathogens in Turkey and Iraq

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The aim of this study was to detect the pathogenic bacteria from hard ticks collected from animals and soil in Turkey and Iraq. For this, 195 adult hard ticks were identified according to the taxonomic keys. A total of 195 hard ticks were identified by as 149 *Hyalomma* spp. and 46 *Rhipicephalus* spp. The diagnosis of tick borne pathogens was made by microbiological techniques. One hundred sixty four (164) bacteria were isolated from the ticks. These bacteria were as follows: 119 *Escherichia coli*, 18 *Salmonella* spp., 10 *Klebsiella* spp., 9 *Serratia* spp., 6 *Shigella* spp., and 2 *Enerobacter aerogenes*. The study investigated tick borne pathogens affecting humans and animals. These pathogens are transmitted by different hard ticks' species. The ticks and tick borne diseases are an important public health problem in the world.

Key words: Hard ticks, *Hyalomma* species, *Rhipicephalus* species, tick borne pathogens.

INTRODUCTION

Hard ticks are obligate blood-feeding ectoparasites which transmit a greater variety of pathogenic microorganisms and are the cause of significant economic losses (Ostfeld et al., 2006). There is great variation in the dominant tick species in different regions of the United States (Merten and Durden, 2000; Stromdahl and Hickling, 2012). Each species of human-biting hard ticks is a vector for a different suite of infectious agents. They can carry different pathogens, and cause important human diseases (Parola and Raoult, 2001). There are several important pieces of hard tick's life cycle. First, at each of the feeding times, obtaining a blood meal is essential for the tick's survival. If the tick fails to find a host at any of these important developmental times, it does not enter the next stage of development and dies as a result; and this has interesting implications for vector control. If one of the host populations were theoretically wiped out, the ticks would not be able to obtain a blood meal and would

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therefore die. Another thing to note about the life cycle is that each tick feeds on different animals, but humans can be infected at any of the feeding stages of a tick. So although ticks generally feed on domestic and wild animals, they are able to feed on humans and that facilitates disease spread (Heaney, 2012).

Hard ticks contain 702 species in 14 genera (Barker and Murrell, 2004). In this study, Genus *Hyalomma* and Genus *Rhipicephalus* were identified. The Genera are common in Asia, Europe, and Africa. These hard ticks are of medical, veterinary, and economic importance because they are the vector of a lot of these pathogens. They transmit the pathogens that cause the animal and human diseases such as, east coast fever, anaplasmosis, babesiosis, rickettsiosis, boutonneuse fever, Lyme disease, Q-fever, Rocky Mountain spotted fever, and crimean-congo hemorrhagic fever, and several bacterial pathogens (Olwoch et al., 2007).

The purpose of this study was to isolate and identify the pathogenic bacteria from hard ticks collected from Turkey and Iraq.

MATERIALS AND METHODS

Design and samples collection

This study applies a descriptive method. One-hundred and ninety five (195) samples of adult ticks were collected from animals and soil from Iraq (Duhok city and Sinjar town) and Turkey (Kahramanmaras city) between the period from April to June 2014. The adult ticks were collected from the back leg and tail regions of the animals and were sent to Kahramanmaraş Sütçü Imam University Medicine Faculty Research Laboratory for the determination of species and for pathogen analysis.

Microbiological and parasitological analysis

For this, 195 ticks were identified under the stereo microscope according to the taxonomic keys and classification criteria (such as capitulum, palp, festoon, cervical canal, anal shield, accessorial shield, and scutum colour) (Dumler and Rosen-Feld, 2000; Walker et al., 2003). Each tick was washed in sterile salty water (0.85% NaCl) and later their outside sections was disinfected with 70% ethyl alcohol, and they were washed in salty water again. Each tick was placed into tubes with sterile distilled water. Then the tubes were homogenized with the homogenizator (Daihan HS-30E, DAIHAN Scientific, Korea) at 3000 rpm for 3 min and then were centrifuged at 4000 rpm for 30 min for extraction of supernatants (Arıkan et al., 2009; Stojek and Dutkiewicz, 2004). Each supernatant was inoculated onto 5% sheep blood agar (Merck, Germany) and endo agar (Merck) agar. Then each culture was incubated in aerobic and anaerobic media at 37°C for 24-48 h. The cultures were evaluated with routine microbiological methods (Dumler and Rosen-Feld, 2000; Koneman et al., 2006).

RESULTS AND DISCUSSION

A total of 195 hard ticks (149 *Hyalomma* spp. and 46 *Rhipicephalus* spp.) were collected from 90 Iraq (46.15%) and 105 Turkey (53.84%) and were collected from different sources like sheep, dogs, tortoises and soil (Table 1, Figure 1). We identified the two genera of the hard ticks by using microscope, according to the external features, presence or absence of festoon, cervical canal, anal shield, accessorial shield, and scutum colour (Figure 2). Our results show that, out of 195 samples which were collected 164 (84.10%) were positive for bacterial growth while 31 (15.89%) samples negative. We isolated six species of bacteria which were characterized and belong to the family of Enterobacteriaceae. The species were *E. coli*, *Salmonella* spp., *Klebsiella* spp., *Shigella* spp., *Enerobacter aerogenes* and *Serratia* spp. Distribution of pathogenic microorganisms isolated from the hard ticks are shown in the Table 2.

The *Hyalomma* spp. bites cause stress and blood loss to the hosts. A few ticks are usually well tolerated by livestock and pets, but infestations with dozens or hundreds of ticks can significantly weaken affected animals and cause weight loss, reduced fertility, and decreased milk production. Several characteristics of the ticks make them abeyance vectors of pathogenic agents. Their wide host range and tendency to feed on several hosts during their life cycle ensures ample opportunity to acquire and transmit pathogens. They have a high reproductive potential, ensuring maintenance of large populations and a high frequency of host-vector contact (Brown, 2005).

The *Rhipicephalus* spp. is the most widespread tick in the world and recognized as a vector of many pathogens affecting dogs, sheep and humans. This tick can be found animals living in both urban and rural areas, being highly adapted to live within human dwellings and being active throughout the year not only in tropical and subtropical regions, but also in some temperate areas (Dantas-Torres, 2010).

These hard ticks represent the most important group of arthropod vectors for wild and domestic animals and also to human; they transmit a wide spectrum of pathogenic microorganisms such as viruses, bacteria, and protozoa (Uilenberg, 1995; Jongejan and Uilenberg, 2004).

In our study, the most commonly isolated bacterium from several species of hard ticks was *E. coli* (72.56%). In consistence with our results, *E. coli* was the most commonly isolated bacteria from sheep in a study in Basra, Iraq (Mohanad and Moaed, 2012). Their study included isolation of ticks from ear, tail and udder of 60 sheep.

The tick samples were identified and assigned to type *Hyalomma* spp. depending on diagnostic characters which included; being festoons or none, legs appeared like banded and shape of spiracle like long coma in male, triangular shape in female with like tail inside at the end.

In 2013, we had conducted another study which showed *Bacillus* spp. as the most commonly isolated pathogen from *Hyalomma* spp. collected from tortoises (Kirecci et al., 2013). In a study, total of 169 ticks, 35

Table 1. Distribution of males and females of the genera of hard ticks.

Genus	Females: n (%)	Males: n (%)
Rhipicephalus spp.	25 (54.34%)	21 (45.65%)
Hyalomma spp.	21 (14.09%)	128 (85.90%)

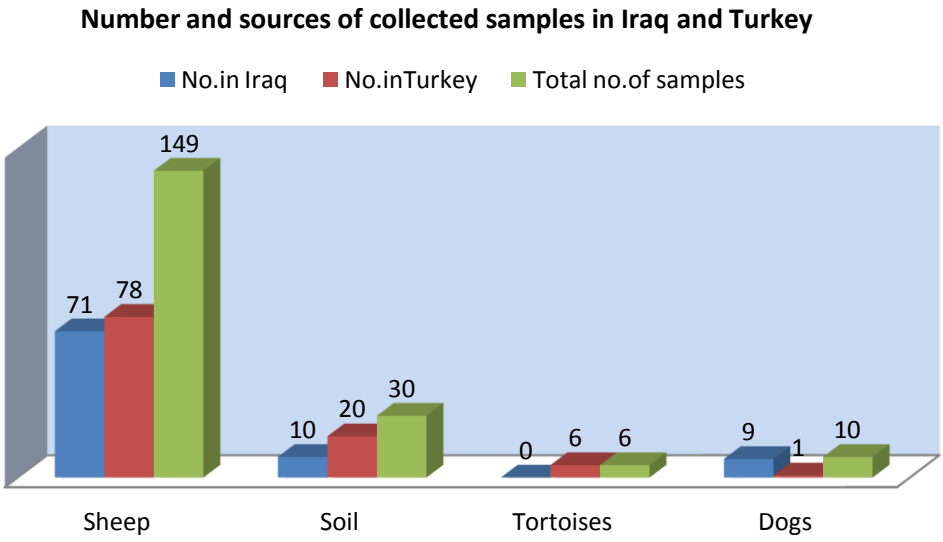

Figure 1. Distribution of the samples of hard tick.

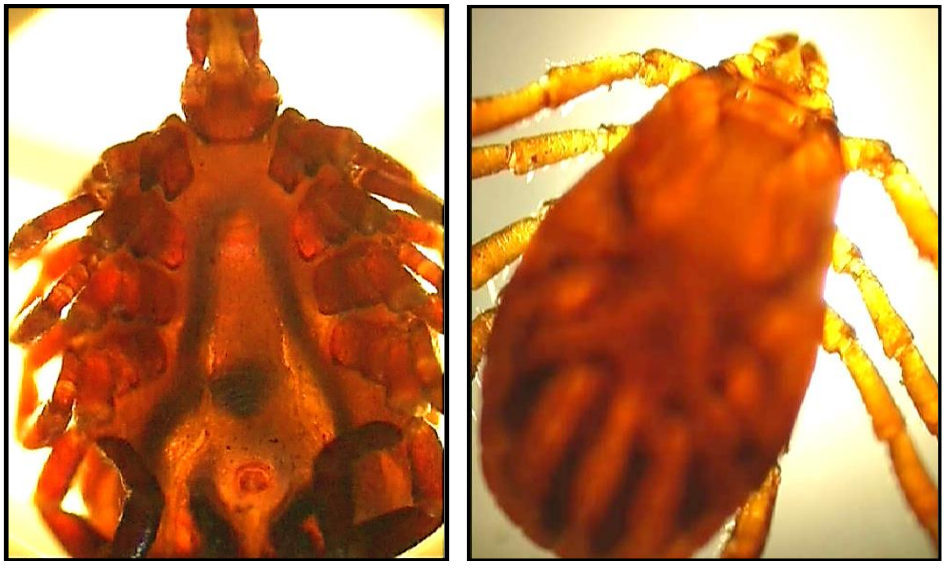

Figure 2. *Hyalomma* spp .(left, adult female, ventral view) and *Rhipicephalus* spp. (right, adult male, dorsal view).

Haemaphysalis parva, 30 *Hyalomma marginatum*, 28 *Rhipicephalus turanicus*, 25 *Dermacentor marginatus*, 17 *H. excavatum*, *16 H. aegyptium*, 6 *Hyalomma* spp. (nymphs), 3 *Ha. punctata*, 3 *Rh. bursa*, 3 *Rh.*

Table 2. Distribution of pathogenic bacteria isolated from hard ticks.

Isolated pathogens from hard ticks	Genera of hard ticks	
	***Rhipicephalus* spp. n (%)**	***Hyalomma* spp. n (%)**
Escherichia coli	22 (13.41%)	97 (59.15%)
Salmonella spp.	8 (4.88%)	10 (6.09%)
Klebsiella spp.	3 (1.83%)	7 (4.27%)
Serratia spp	3 (1.83%)	6 (3.66%)
Shigella spp.	1 (0.61%)	5 (3.05%)
Enterobacter aerogenes	1 (0.61%)	1 (0.61%)
Total = 164 (100%)	38 (23.17%)	126 (76.83%)

sanguineus, 2 *Ixodes ricinus*, and 1 *Haemaphysalis* spp. (nymph), were collected from humans in different parts of Ankara, in Turkey (Orkun et al., 2014). In our study, a total of 195 hard ticks, 149 *Hyalomma* spp. (adult) and 46 *Rhipicephalus* spp. (adult) were collected from sheep, dogs, tortoises and soil in Kahramanmaraş city of Turkey and in Duhok city of Iraq. A surveillance study that lasted 4 years in the US categorized 66. 000 types of ticks and *Rickettsia* spp., an important pathogenic bacterial species, were identified in these ticks (Merten and Durden, 2000).

Hard ticks can be vectors of human diseases such as CCHV, tick paralysis, and Lyme disease. In our study, viruses and unculturable pathogens were not investigated, however, the bacteria we determined are very important pathogens for humans; *Salmonella* spp. and *Shigella* spp. can cause gastroenteritis in humans, while other species may lead to sepsis and various soft tissue diseases.

The prevalence of zoonoses and other infections have been increasing because of expanding human migrations and global climate change. Tick borne pathogens are particularly dangerous in countries with a temperate climate and serious preventive measures should be taken against these infections.

This study agrees with the study of Heaney (2012) since his study shown the importance of ticks in transferring diseases to humans and animals and this importance include the three important steps; first, they can feed on a host animal that is infected and ingest the pathogen in the host's blood. Second, ticks can perform transovarial transmission for most diseases. This means that mothers can pass on the pathogen to their offspring. Third, co-feeding, or multiple ticks feeding at once on the same host, can cause infection. Thus, ticks can carry diseases at every point of their lives and each time a tick needs a blood meal, humans are at risk for catching the disease.

Ticks of the genus *Hyalomma* spp. are known as a vector of viruses such as Congo-Hemorrhagic Fever (CCHFV). Although many species are not involved in disease transmission, the considerable length of *Hyalomma* spp. mouthparts provokes a painful bite. One of the most important diseases transmitted by this ticks is CCHFV which occurs throughout vast area of Africa, Asia and Europe, but can cause high mortality (Sadek et al., 2007).

To conclude, hard ticks are the most dangerous arthropod which impend or threaten health of vertebrates and they are capable of transmitting the greatest variety of pathogens for both humans and animals and the riskier of hard ticks lies through their feeding manner that makes them important in the health of domestic animals and humans.

Conflict of interest

Authors have not declared any conflict of interest.

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Full Length Research Paper

Inoculation of *Bradyrhizobium* with cellular additives and micronutrients in soybean seeds cultivated in Oxisol under no-tillage system

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In the bid to get increased yield of soybean, several studies on biological fixation of nitrogen have been done in order to enhance the results of this technology with cellular additives and micronutrients. The aim of this study was to evaluate the use of inoculant (*Bradyrhizobium japonicum* SEMIA and 5019) associated with cellular additives, cobalt and molybdenum (Fertiactyl® leg) in soybeans seeds in Oxisol cultivated under no-tillage system. The experiment was conducted in a randomized block with two treatments (with and without commercial product) and 10 repetitions, totaling 20 experimental plots in the cultivation of soybean. The experiment was done in 2011/2012 and 2012/2013. We evaluated the N content in grain yield and weight of 100 grain in the 2011/2012 and grain yield, weight of 100 grain and foliar content of N, Ca, Mg, K, P and S in the 2012/2013 crop. Inoculation of the *B. japonicum* with cellular additives, cobalt and molybdenum shows greater accumulation of N in soybean grain, but does not influence the weight of 100 grains and yield in the culture of soybean in 2011/2012 and 2012/2013. There was an increase of Cu and reduction of K, P and S in the leaf tissue of soybeans with the presence of the *B. japonicum* with cellular additives, cobalt and molybdenum (Fertiactyl® leg). It is recommended that farmers should not sow soybean with seed treated with fungicide and insecticide for 12 h together with *B. japonicum* inoculation, cellular additives, Co and Mo; they cause nutritional changes without interfering in the yield under no-tillage in Oxisoil.

Key words: *Bradyrhizobium japonicum*, *Glycine max*, seed treatment, glifosate, fungicide, insecticide.

INTRODUCTION

Soybean is one of the most economically important crops in the world, as the area under cultivation is the expressiveness of production (USDA, 2013). Indeed it is one of the most cultivated crops in the world, used

primarily as a source of protein for human and animal (Graham and Vance, 2003; USDA, 2013).

The interest in increasing production is no longer focused on increasing crop areas, but the increase in production capacity in the same area. This is favored by technologies such as fertilization with biofertilizer (Moreira and Zibetti, 2011), soluble, reactive and natural phosphates (Luchini et al., 2012), poultry litter (Piano and Seidel, 2012).

Nevertheless, the increasing potential of production in the world also requires adopting effective and economically viable ways to availability of nitrogen (N) for grain production (Zilli et al., 2006, 2010b). This is because approximately 40% of soybeans are composed of proteins (Embrapa, 2011; Rodrigues and Silva, 2011), which explains the high demand of this nutrient.

In Brazil, nitrogen fertilizer in soybean is unusual, being the biological nitrogen fixation (BNF) responsible for the provision of most of the nutrients needed to produce and achieve high yield (Embrapa, 2003; Zilli et al., 2006; Embrapa, 2011). In the 1980s, Vargas et al. (1982) stated that BNF could sustain grain yield of up to 4 t ha^{-1}. Since most soils cultivated with soybeans in Brazil belong to the class of Oxisols with low levels of soil organic matter (OM), consequetely low N, appropiate agronomic practices can increase the efficiency of BNF and increase yield (Pauferro et al., 2010).

Soil management can also interfere with temperature, moisture, nutrients, organic matter and crop management to increase the efficiency of N-fixing bacteria (Campos and Gnatta, 2006; Lucca and Hungria, 2014) and provide increased yield in soybean. Consolidated no-tillage systems have soils with lower rate of oxidation of organic matter and consequently a higher content of organic matter, mainly by the presence of soil cover. The result is the maintenance of higher moisture and lower thermal variation in the soil, and the availability of nutrients, reducing the need for fertilizers. It also favors the increase and maintenance of the populations of microorganisms in the soil, especially nitrogen-fixing bacteria (Zilli et al., 2006).

The process of BNF in Brazil is responsible for nitrogen accumulated by plants; it represents about 200 kg ha^{-1} N (Zilli et al., 2010b), which is no longer applied via mineral fertilizers. This reduces the cost of production (Albareda et al., 2009). Thus the application reduces too much nitrogen fertilizer, which can contaminate water tables (Jadoski et al., 2010; Macdonald et al., 2011). The overuse of mineral fertilizers, such as nitrogen has caused environmental problems in some parts of the planet. In Europe, environmental costs including all nitrogen losses were recently estimated at 70-320 billion euros per year, which exceeds the direct economic benefits of N in agriculture (Foley et al., 2011).

The efficiency of BNF, in turn, is dependent not only bacterial strains used and symbiosis with culture, but on adequate availability of certain chemical elements. Cobalt (Co) and molybdenum (Mo) are essential for BNF (Taiz and Zeiger, 2013). The first B12 vitamin is essential for the processing of BNF and other parts of the molibdo-enzymes, used in absorption and metabolism of nitrogen (Novais et al., 2007).

The processing of BNF can reduce the pH of the soil, especially next to the root system of the plants. This reduction is responsible for the increased absorption of some nutrients in the soil, like iron (Fe) (Souza et al., 2010). These authors mention that by the time it becomes effective symbiosis, soybean plants may exhibit Fe deficiency, making it not observed later with the low pH in the rhizosphere. According to Silva et al. (2011a), the survival of bacteria can vary depending on the pH, salinity, and bactericidal action of some products, to achieve the application of Mo and Co with *Bradyrhizobium* sp. in soybean seeds.

However, studies have shown the incompatibility between *Bradyrhizobium* sp. and practices of seed treatment with fungicides and insecticides in soybean (Campo and Hungria, 1999; Bueno et al., 2003; Zilli et al., 2010a; Pereira et al., 2010; Embrapa, 2011; Marks et al., 2013; Favero and Lana, 2014). In this sense, Tedesco and Campos (2000), citing inoculants 2000s, recommended that inoculated soybean seeds should not be stored for more than a day, so the suggested level of viable cells per seed *B. japonicum* is reached. In the search for new technologies and product formulations to enhance seed treatment with *Bradyrhizobium* sp., cellular additives were tested to enable greater concentration of live microorganisms in seeds during inoculation process (Marks et al., 2013). This enables performing advance inoculation before sowing.

Thus, the objective of this study was to evaluate the use of inoculant (*B. japonicum*) associated with cellular additives, cobalt and molybdenum in soybean seeds in Oxisoil grown in no-tillage system.

MATERIALS AND METHODS

The work was executed in the West of the State of Paraná, Brazil, in soil classified as Oxisoil very clayey (Embrapa, 2013) with the following coordinates: 24º15'S and 54°10'W and altitude of 338 m (Figure 1). The farm has cultivated soybeans for 30 years and used no-tillage for 20 years in succession of crops, using soybean in summer and wheat/corn in the winter. Before the experiment, the area was occupied by wheat crop.

The soil granulometric characteristics of the property are 650 g kg^{-1} clay, 130 g kg^{-1} sand and 220 g kg^{-1} silt, and the result of the

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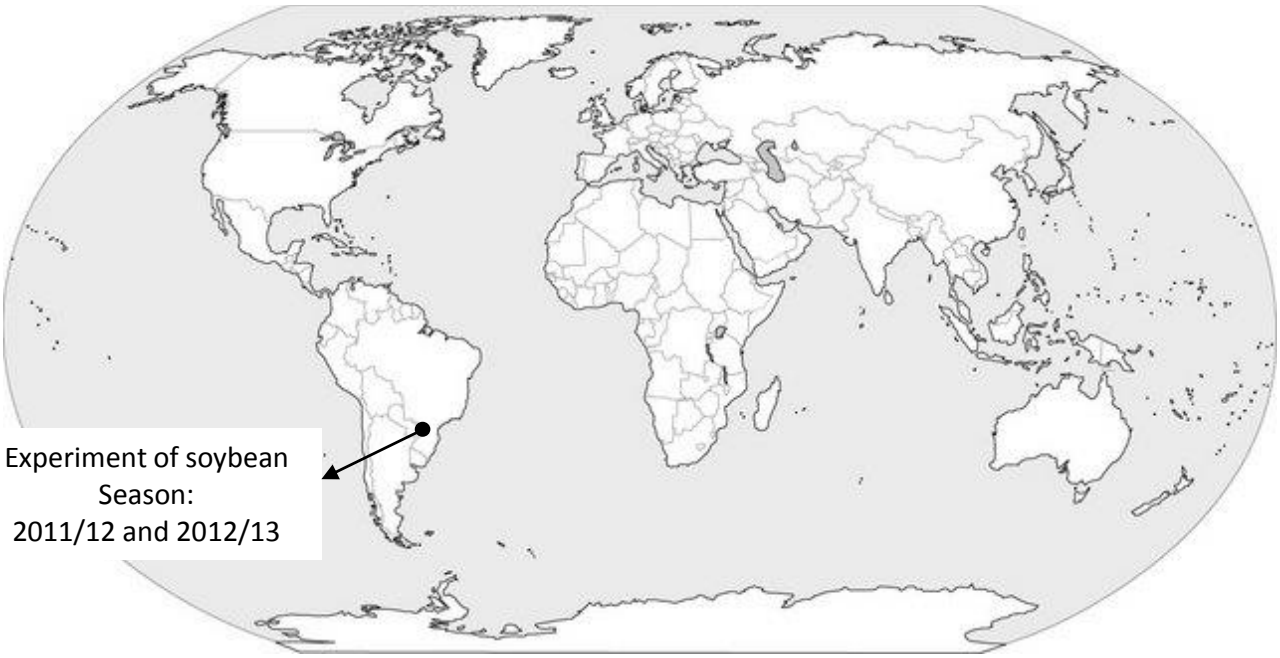

Figure 1. West of Paraná state, Brazil.

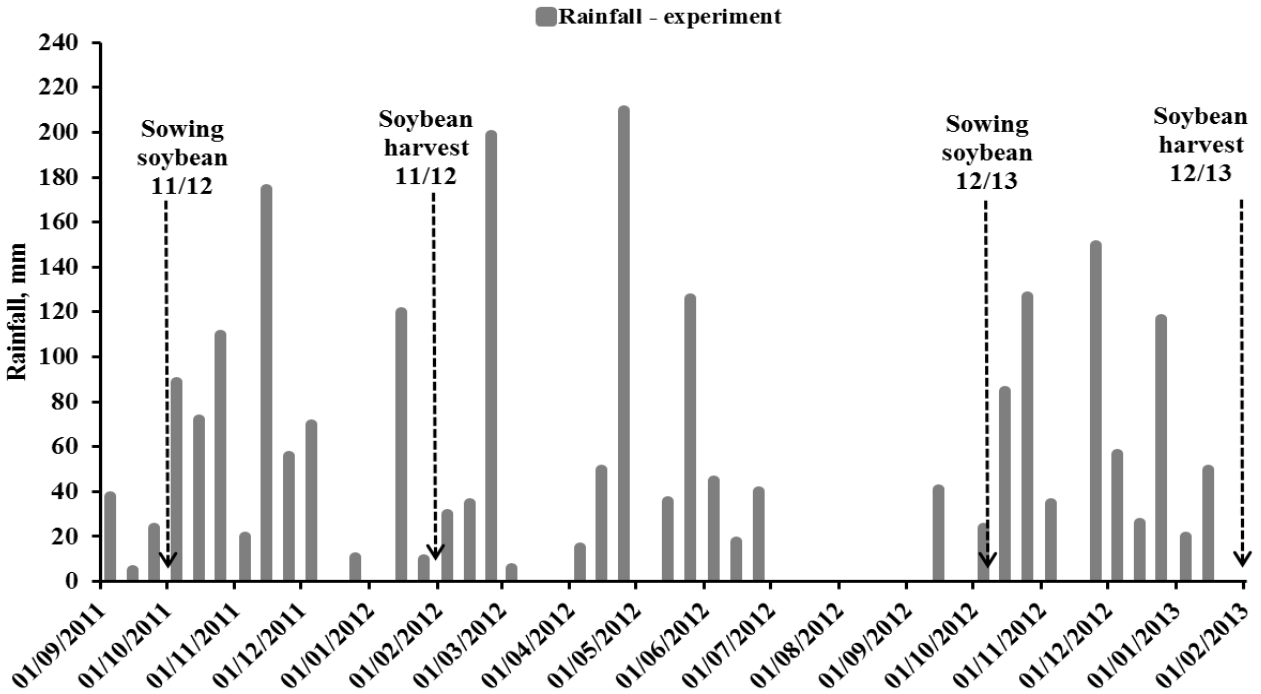

Figure 2. Rainfall accumulated (mm) every ten days in the experimental area during the driving period between 09/01/2011 to 02/01/2013.

chemical analysis gives the following values: pH in $CaCl_2$ = 4.80;C = 16.70 g dm^{-3}; P = 12.40 mg dm^{-3}; K^{+} = 0.20 $cmol_c$ dm^{-3}; Ca^{+2} = 4.67 $cmol_c$ dm^{-3}; Mg^{+2} = 1.64 $cmol_c$ dm^{-3}; H+Al = 5.76 $cmol_c$ dm^{-3}; Al^{+3} = 0 $cmol_c$ dm^{-3}; SB = 6.51 $cmol_c$ dm^{-3}; CEC = 12.27 $cmol_c$ dm^{-3}; e V% = 53.06. At this location, the accumulated rainfall every ten days recorded during the experiment is shown in Figure 2 and second Koppen, the climate is Cfa, subtropical with rains well distributed throughout the year and hot summers (Caviglione et al., 2000). The rainfall for the first season of soybean in 2011/2012 at sowing to harvest was 733 mm and in 2012/201313, it was 621 mm.

The experiment was arranged in randomized complete block design with two treatments and 10 replications, totaling 20 experimental plots in two seasons (2011/12 and 2012/13). The treatments consisted of control with no inoculation and cobalt (Co) and molybdenum (Mo) and treatment Fertiactyl leg ® [*B. japonicum* SEMIA 587 and 5019 inoculant with Extender (additive cellular), to keep the bacteria viable for long, with cobalt (Co) and molybdenum (Mo) and natural sources of amino acids, humic and fulvic acids] (Timac Agro Brazil, 2013) with double dose due to pH = 4.80 $CaCl_2$ soil used. In both seasons, the soybean seed treatment was performed 12 h before sowing. In the same manner, two cultivations were carried out with seed treated with fungicides fludioxonil (25 g L^{-1}) + metalaxyl – M (10 g L^{-1}), in the recommendation of 100 mL per 100 kg of seed, and fipronil (250 g L^{-1}) with 200 mL per 100 kg of seed (EMBRAPA, 2011).

In both seasons, ten days before sowing ghyfosate herbicide (3 l ha^{-1} Roundup Ready) and ghyfosate herbicide in V5 stage (1.5 l ha^{-1} Roundup Ready) were applied. It was first deployed soybean cultivar with Vmax RR (SYN 7059RR) on October 2011, in summer 2011/2012 season for assessment in first year, with 14 plants per meter. During the 2012 winter crop sowing corn P3340HX, which is recommended for the region as agroclimatic zoning of Paraná, was performed. Subsequently, treatments were tested in the cultivation of summer crops 2012/2013, with the sowing of transgenic soybean cultivar Potencia RR on October 2012 (Embrapa, 2011), with 14 plants per meter. The use of different cultivars was to alter the genetic material and reduce pest problems related with soil conditions that occur with the use of successive soybean in summer.

During period of the experiment we performed, soybean sowing in 0.45 m spacing, density of 15 seeds per meter and final stand of 266,666 plants ha^{-1} in both summer crops. The plots were total area of 12.00 m^2 and usable area of 5.40 m^2. Monitoring of pests, diseases and weeds and need for control was performed according to the recommendations for soybean (Embrapa, 2011) and for maize cultures (Rodrigues and Silva, 2011). The sowing of soybean was performed with precision seed drill Marchesan Ultra Flex with 11 soybean lines coupled to tractor.

The fertilization of soybean crop in 2011/2012 250 kg ha^{-1} of superphosphate fertilizer was used with 19% P_2O_5. In the second crop corn harvest 250 kg ha^{-1} was used as fertilizer formulated 16-16-16. The next crop of soybeans in the 2012/2013 crop, 250 kg ha^{-1} of this fertilizer 4-15-10 was used with 25% OM (organic matter), Minorgan Organofós (Minorgan, 2013).

In the 2011/2012 harvesting, grains collected had weight of 100 (M 100), and the levels of nitrogen (N) of grains were also determined.

In the second cropping of soybeans in the 2012/2013, leaf samples were collected in full bloom as third trifoliate, as recommended procedures to time and sample leaf described by Malavolta et al. (1997).

Soybean leaves collected were dried in a forced-air oven for three days at 65 ± 2°C, ground to smaller size and analyzed. First, 0.02 g of dry mass was digested by 4 mL HNO_3+$HClO_4$ (3:1) for determining K, Ca, Mg, Cu, Fe, Mn and Zn. Concentrations of Ca, Mg, Cu, Fe, Mn and Zn were determined by flame atomic absorption spectrophotometry, K determined by flame photometer, P and S were determined by colorimetry, as previously described by Embrapa (2009). Concentrations of N in grains in 2011/2012 and leaves in 2012/2013 were determined by 2 mL H_2SO_4 digestion of 0.02 g dry mass and vapor distillation of Kjeldahl (Tedesco et al., 1995).

At the point of harvesting of crop, on February 5, 2013 in the 2012/2013, plants were collected at the weight of 100 grains (M 100) and yield. In both seasons, plants were threshed on "Vencedora B-150" to obtain the grains, for determining the weight of grains and standardizing the moisture content of the samples to 14%.

Table 1. Nitrogen content in grain (grain N), weight of 100 grains (M 100) and soybean yield in the 2011/2012 season based on the presence and absence of Fertiactyl leg® (*Bradyrhizobium japonicum* inoculant with Extender, to keep the bacteria viable for more time and with cobalt (Co) and molybdenum (Mo), with natural sources of amino acids, humic and fulvic acids) grown on Oxisol in the city of Terra Roxa/PR, 2012.

Treatment	Soybean (2011/2012)		
	N grain	M 100	Yield
	g kg^{-1}	g	kg ha^{-1}
Witness	17.72b	10.42	1,170.70
Inoculant	24.63a	10.30	1,191.50
Source of variation		F Values	
Treatments	5.03 *	0.23 ^{ns}	0.36 ^{ns}
CV (%)	32.52	5.33	6.54

*Significant at 5% by F test; ^{ns}not significant at the 5% level by F test.

Statistical analysis of the results was performed with the GENES program (Cruz, 2006), so the data were subjected to analysis of variance. In case of significant effects, we used the actual F-test to verify the probability of a significant difference between the means, because with only one degree of freedom for the source of variation, the F test is conclusive.

RESULTS AND DISCUSSION

The inoculation with *B. japonicum* in addition to fertilization with cobalt and molybdenum showed greater accumulation of N in grain of soybeans in 2011/2012 harvest, differing ($P<0.05$) from the control. However, this was not reflected in greater mass of 100 grain and crop yield (Table 1). Meschede et al. (2004) reported that seed treatment with cobalt (Co) and molybdenum (Mo) improved seed quality in relation to protein content, that is related with N content in grain. Albareda et al. (2009), Zilli et al. (2010a) and Favero and Lana (2014) also observed that positive effects of inoculation increased levels of N in soybeans, when seeds were inoculated with *B. japonicum* without seed treatment while Lantmann et al. (1989) observed increases of 3-6% of protein in grains, when Mo was used in soybean. Diesel et al. (2010) did not find significant results with application of foliar molybdenum and cobalt for the weight of 100 grains.

The absence of effects on the weight of 100 grains and yield with inoculation was also observed by Silva et al. (2011a) regardless of inoculum dose and way of application of Co and Mo in the first crop. Pessoa et al. (1999) in an Oxisol also observed no interference of Mo foliar application in *B. japonicum* inoculation. On the other hand, Hungria et al. (2013) related problems of inoculation with *B. japonicum* on seed of soybean with fungicide

Table 2. Weight of 100 grains (M 100) and soybean yield in the 2012/13 season based on the presence and absence of Fertiactyl leg® (*Bradyrhizobium japonicum* inoculant with Extender, to keep the bacteria viable for more time and with cobalt (Co) and molybdenum (Mo), with natural sources of amino acids, humic and fulvic acids) grown on Oxisol in the city of Terra Roxa/PR, 2012.

Treatment	Soybean (2012/2013)	
	M 100 (g)	Yield (kg ha^{-1})
Witness	15.41	4,290.56
Inoculant	15.45	4,292.44
Source of variation	F Values	
Treatments	0.01^{ns}	0.01^{ns}
CV (%)	4.92	4.62

*Significant at 5% by F test; ^{ns}not significant at the 5% level by F test.

and insecticide, in minimizing favorable effects of biological nitrogen fixation, and research possibility by in-forrow inoculation.

The inoculation of *B. japonicum* using fertilization with Co and Mo for soybean crop in 2012/2013 did not influence variable mass of 100 grains and yield (Table 2) in clayey Oxisol at pH 4.80 for $CaCl_2$. Silva et al. (2011a, b), who grow soybeans in dystrophic typic with a pH $CaCl_2$ of 4.6 with V% of 40, found different results. In the second year of cultivation, the application of Co and Mo in soybean increased the mass 100 grains, irrespective of the method of application, but, there was no increase in grain yield. Similarly, Silva et al. (2011b) reported that the weight of 100 grains is a characteristic value of each cultivar, however, does not prevent difference with changes of environmental and/or management to which the culture is subjected. In this research, in both seasons glifosate herbicide was applied before sowing and V5 stage of soybean. It is an important information, because recent research shows that BNF and yield parameters were more affected by location, cropping season and cultivar than by the applied glifosate herbicide in the culture or weed-management strategy (Nakatani et al., 2014; Hungria et al., 2014). Glifosate did not interfere in the results, therefore, Malty et al. (2006) did not see any effect on nodulation in the study with doses of glifosate applied before sowing soybean.

Meschede et al. (2004) said the expected response to Mo and Co in soybean nodulation is lower in soils with high organic matter content, high fertility and acidity adjusted. So much so that Lantmann (2002) found greater responses to the application of Mo in conditions of pH $CaCl_2$ less than 4.3, for Latosolic Alic, and less than 4.8 for the Oxisol Dark Alic. However, Marcondes and Caires (2005) found that application of Mo and Co in the seed grown in soil with pH $CaCl_2$ 5.2 did not affect nodulation of soybean plants. Thus, the pH may be less relevant when it is growing under no-tillage for several years (Pessoa et al., 1999).

On the other hand, an increase in yield was observed by Zilli et al. (2010a) and with a low content of organic matter in soil and the soil pH $CaCl_2$ of 5.2. In addition, Barbaro et al. (2009) observed response of soybean cultivars to inoculation and application of cobalt and molybdenum in pasture areas of reform. The application of molybdenum and cobalt via seeds and/or the V4 leaf stage provided a significant increase in soybean yield, with increases of up to 240 kg ha^{-1} in grain production in the culture area of reform pasture in dystrophic Red-Dark texture sandy $CaCl_2$ at pH 5.2. The agronomic parameters evaluated were positively affected by the application of Co and Mo even in Red Hapludox clayey with pH $CaCl_2$ 4.4, particularly when applied as a foliar both by seed (TS + V4), including grain yield. The way of application was not significantly different, that is both the application via seed and foliar were efficient in providing these nutrients for soybean (Dourado Neto et al., 2012). Recently, Favero et al. (2013) found that the use of *B. japonicum* inoculation + Co and Mo together in seed treatment with fungicide and insecticide increased the yield of soybean, as observed reduction in plants with "crazy soybean II" in soil with pH 4.7. It affected plants record, high abortion rate of flowers and pods, which prevents natural maturation of the plant, and remains green even after desiccating herbicides. According to the authors, these results may have been evidenced due to the low pH of the soil, which reduces the availability of Mo on the ground, damaging the fixation of N_2 in soil.

Other researchers, like Campos (1999) also found similar results in their survey in Typic dark, with five years in no-tillage, where there was no significant effect on grain yield of soybean with application of up to 1 kg of peat inoculant for each 50 kg seeds. Pessoa et al. (1999), in an experiment conducted on an Oxisol in pH H_2O at 5.3, said no response was observed for seed treatment with *B. japonicum* and Mo or when it was used in foliar fertilization. Chueiri et al. (2005), in areas with high acidity and pH less than 5.5 in water, recommend applying double dose of inoculant *B. japonicum*, because the acidity of the soil interferes with the survival of bacteria and also increases competition among native strains and selection. Therefore, it is expected that this increases the amount of inoculant to compensate for the loss of viable cells.

The difference in soybean yield obtained in 2011/2012 (Table 1) and 2012/2013 seasons (Table 2) is probably related to rainfall condition in every culture since in the first and second crops, prolonged drought was satisfactory rainfall conditions for the development of culture (Figure 1). Water is necessary for increasing microbiology activity and for photosynthesis (Taiz and Zeiger, 2013); also, growth is sustained by nitrogen fixation rates and photosynthesis activity. Luca and

Table 3. Levels of nitrogen (N), potassium (K), phosphorus (P), calcium (Ca), magnesium (Mg) and sulfur (S) in soybean leaves in the 2012/13 season based on the presence and absence of Fertiactyl leg® (*Bradyrhizobium japonicum* inoculant with Extender, to keep the bacteria viable for more time and with cobalt (Co) and molybdenum (Mo), with natural sources of amino acids, humic and fulvic acids) grown on Oxisol in the city of Terra Roxa/PR, 2013

Treatment	N	K	P	Ca	Mg	S
	g kg^{-1}					
Witness	51.20	13.40 a	3.68 a	10.46	2.12	12.04 a
Inoculant	51.60	11.53 b	3.16 b	9.66	2.35	10.91 b
Source of variation			F Values			
Treatments	0.22^{ns}	8.09*	6.78*	3.31^{ns}	0.21^{ns}	4.99*
CV (%)	3.59	11.76	13.09	11.98	49.96	9.90

*Significant at 5% by F test; ^{ns}: not significant at the 5% level by F test.

Hungria (2014) related that there are lower plants of soybean in areas of quadruple photosynthesis and nitrogen fixation rates. In this research, 14 plants per meter can be reduced; therefore, the efficiency of water use present in the soil was elevated mainly in 2011/2012.

In general, lack of response to the addition of chemical elements Co and Mo may be associated with adequate levels of availability in the soil or seed with sufficient concentrations of the need for culture (Ishizuka, 1982). In fact, Pessoa et al. (1999) found that the source of basalt soil, rich in micronutrients, with high fertility, acidity corrected by liming and pH $CaCl_2$ 5.3 in no tillage system probably has Mo enough to meet the requirement of soybean.

As reported by Gris et al. (2005) lack of response between treated and untreated seeds with inoculant is likely to occur in soil that already has established populations of bacteria that provide adequate nodulation. As the place used in this work has grown soybean for the last 30 years, there is likely going to be the establishment of populations of nitrogen fixing bactéria. Thus, the increase in yield resulting from inoculation in areas already previously cultivated with soybeans, are less expressive than in areas of first year (Campos, 1999; Campos and Gnatta, 2006). However, average gain of 4.5% was observed in grain yield with inoculation already cultivated areas (Embrapa, 2003).

Critical periods of leguminous vegetable other than the nitrogen biological fixation of nitrogen have been reported.

For example, at the early stage of plant growth when symbiosis is being developed, the N-fixed cannot supply the amount of N to meet the demand of the plant (Gan et al, 2003). In addition, when the culture is achieved, the filling phase of pods can result in nodule senescence. In these situations, and when the supply of soil N is not high enough, one option would be to supplement it with mineral N fertilizer of leaves.

Regarding the contents of macronutrients in leaves of soybean evaluated, only K, P and S (Table 3) decreased with inoculation with Co and Mo, specifically in the 2012/2013 harvest. Vieira Neto et al. (2008) actually found a low effect on levels of macronutrients N, P and S in leaves of soybean. Tiritan et al. (2007) observed 6% increase in the content of S, Mo and Co in the seed.

The reduction in the contents of K, P and S in the leaf tissue of soybean inoculated may be related to the supply of N to the roots of soybean inoculated (Gris et al., 2005). Intensifying the metabolic activity in roots can increase the demand for P and K by this organ. Different results were found by Marcondes and Caires (2005) that the levels of macronutrients in leaves of soybean were slightly influenced by the application of molybdenum in the seeds.

Vieira Neto et al. (2008) working with soybean plants obtained from seeds subjected to different treatments of inoculation, noted that there was no difference between treatments for N, P and S; however, the K observed that treatment (fertilization with N-fertilizer (200 kg ha^{-1}) was lower than the others.

Lack of effect of leaf N (Table 3) was also observed by Toledo et al. (2010) who found no interference with the values obtained by inoculation with *B. japonicum*. The results corroborate those obtained by Marcondes and Caires (2005), showing that the presence of molybdenum did not change the nitrogen concentration in soybean leaves. Albino and Campo (2001) also found no change in the levels of nitrogen in soybean plants by applying sodium molybdate immediately after inoculation on seeds. Favero and Lana (2014) did not observe effect of leaf N when they inoculated soybean seed with *B. japonicum* + Co + Mo + insecticides + fungicides at the moment of sowing.

Regarding the levels of micronutrient on soybean leaves, only Cu (Table 4) was similar ($P<0.05$) and increased with inoculation together with cobalt and molybdenum used in the 2012/2013 harvest. Vieira Neto et al. (2008) found that micronutrient absorption by soybean plants was not affected by the treatments involving inoculation with *B. japonicum*, showing no

Table 4. Levels of copper (Cu), zinc (Zn), manganese (Mn) and iron (Fe) in soybean leaves in the 2012/13 season based on the presence and absence of Fertiactyl leg® (*Bradrhyzobium japonicum* inoculant with Extender, to keep the bacteria viable for more time and with cobalt (Co) and molybdenum (Mo), with natural sources of amino acids, humic and fulvic acids) grown on Oxisol in the city of Terra Roxa/PR, 2013.

Treatment	Cu	Zn	Mn	Fe
	mg kg^{-1}			
Witness	20.89 b	22.82	31.95	103.19
Inoculant	44.54 a	19.40	31.74	106.32
Source of variation	F Values			
Treatments	7.89 *	0.71 ^{ns}	0.01 ^{ns}	0.16 ^{ns}
CV (%)	57.55	42.76	48.80	16.86

*Significant at 5% by F test; ^{ns}:not significant at the 5% level by F test.

changes in foliar levels of soybean. Marcondes and Caires (2005) also found that the concentrations of Cu, Zn and Mn on soybean leaves were not influenced by the application of molybdenum in the seeds. This is similar to results obtained in this work, except for Cu, which showed a higher content when Fertiactyl leg® was used.

According to Souza et al. (2010), plants fixing N has the ability to reduce the pH of the soil, especially next to the root system of the plants. This reduction is responsible for the increased absorption of some nutrients in the soil. Thus, in the soil the increased absorption of Cu may be associated with organic matter, which retains this nutrient in the form of the inner sphere complex (Novais et al., 2007); it may be available to the soil solution to increase acidity in the rhizosphere provided by the nutrient uptake in soybean crop in the treatment with seed inoculation.

An important factor that deserves to be highlighted is that the inoculation was performed 12 h before sowing with the Fertiactyl ® leg with cellular additives (Timac Agro Brazil, 2013), which serve as food for bacteria to survive up to seven days. Thus, the absence of significant results regarding grain yield may be related to poor survival of bacteria after inoculation (Embrapa, 2011), as sowing was not performed immediately after treatment. Also and mainly because seed treatment with fungicide, evidenced by Zilli et al. (2010a) and Embrapa (2011), who identified inhibition of nodulation of bacteria in this situation, has been performed and also Pereira et al. (2010) observed a reduction in nodulation and growth of soybean plants by seed treatment with carbosulfan insecticide, clothianidin, fipronil, imidacloprid and thiamethoxam depending on the strain used for inoculation. Negative effects have also been observed in the BNF in the associated use of *B. japonicum* with fungicides. Bueno et al. (2003) and Zilli et al. (2010b) tested the effect of fungicides on survival and nodulation of *B. japonicum* and obtained a reduction of microorganisms in combinations of fungicides carboxim+thiram. Moreover, Marks et al. (2013) found that the use of cell additive in combination with inoculant and specifically Maxim fungicidal seed treatment of soybean improved survival and longevity of *B. japonicum* in the seed, and maximized BNF technology.

Another possibility is that these results demonstrated that, probably, the nutritional needs of N by soybean were supplied by the process of symbiotic N_2 fixation, regardless of the use of seed treatment with *B. japonicum* with Co and Mo, as has been cultivated soybean for several years, and the type of soil, minimizing the effects of inoculation. In this situation, it is pertinent to assess new work in the field, with the prior execution of seed treatment fungicides and insecticides, and inoculation with *B. japonicum* with Co and Mo at the time of sowing date. Seeing that the beneficial effects of using insecticides and fungicides for seed treatment is imminent, it is necessary to continue studies to find the ideal setting for inoculation which really identifies the time, since reduction of inoculation may occur to effectuate it in the same instant that the fungicide and insecticide are used. Therefore, recent research shows physiological and yield performance with in-furrow inoculation has been useful alternative for soybean, because it does not use seed treated with fungicides, decreases nodulation (Zilli et al., 2010b) with fungicides/insecticides, does not interfere with on leaf N and yield. Although there is observed green stem and leaf retention in soybean, similarly it uses seed without anything in the sow (Favero and Lana, 2014).

Conclusion

Seeds treated with fungicide and insecticide together with *B. japonicum* inoculation, cellular additives, cobalt and molybdenum (Fertiactyl® leg) used in soybean seeds 12 h before sowing provide greater N accumulation in the grain, increase Cu and reduce K, P e S in leaves, but do not influence the weight of 100 grains and yield in soybean.

It is recommended that farmers should not sow soybean with seeds treated with fungicide and insecticide for 12 hours together with *Bradyrhizobium japonicum* inoculation, cellular additives, Co and Mo; this is because they cause nutritional changes without interfering in the yield under no-tillage in Oxisoil.

Conflict of interests

The authors did not declare any conflict of interest.

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