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ISOLATION AND CHARACTERIZATION OF PROBIOTIC *LACTOBACILLUS* SPECIES FROM CURD SAMPLES AND EVALUATION OF THEIR ANTAGONISTIC POTENTIAL

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ABSTRACT

Fermentative milk products with probiotic properties escalate as a necessary substitution in human and animal feed consumption for well being of the gut microflora. One of the key aspects is being the reducing the population of pathogens and production of vitamins. The live bacterial community of these fermented foods can reduce the effect of antibiotics and increase the immune function. Ten beneficial *Lactobacillus* sp. were isolated from two local curd samples and characterized in terms of morphological, biochemical, molecular and physiological characteristics. Antagonistic potential of all the isolates were studied against enteric pathogens; *Escherichia coli*, *Salmonella typhi*, *Shigella boydii* and *Enterococcus faecalis*. All are gram-positive rod of non-endospore forming ability with pH tolerance range of 5-8, NaCl tolerance limit of 5% (w/v). A wide spectrum of antibiotic resistance was noticed for all the isolates. Almost all isolates showed antagonism against *E. coli* and *S. typhi* followed by *S. boydii* and *E. faecalis* and strains OC3 and KC2 were found to be the best potential candidates. Bacteriocin extract showed a little difference denoting OC3 and KC2 as well as OC5 as effective against all the enteric pathogens. 16S rRNA gene sequence analysis and phylogenetic characterization revealed the taxonomic affiliation of the strain OC3 and KC2 to *L. brevis* ATCC 14869 (T) and *L. paraplantarum* DSM 10667 (T) showing 99% of sequence similarity with high phylogenetic lineage towards probiotic *Lactobacillus* type strains. These strains can be used as potential probiotic agents as starter inoculum for the fermented foods and reducing disease incidence caused by enteric pathogens in human and help in dropping antibiotic consumption.

Keywords: Probiotics, *Lactobacillus* sp., antagonistic activity, bacteriocin, phylogeny

INTRODUCTION

Fermented foods and milk products with health benefits are known to human kind since ancient times and the relationship has been investigated for long years **(Pyar and Peh, 2014)**. Consumption of active microorganisms (probiotics) through fermented food products has been centre of attraction among all. Probiotics are the active microorganisms which when administered orally in required amount confer health benefits **(Asha and Gayatri, 2012)**. Recent research has been focused on exploring potential probiotics candidate in the form of fermented food like yoghurt, cheese, pudding, ice-cream, sausages and other milk products to replace antibiotic based drug intake **(Chan *et al*., 2000)**. Because, recent trend of emergence of antibiotic resistant pathogenic strains with multiple drug resistance (MDR) capacity have become a serious threat to human health **(Ahmed, 2013)**. As a result, there is increasing probability of gastrointestinal infection and high mortality rate. According to World Health Organization (WHO), enteric infection is the fifth leading cause of death among all age groups worldwide and the causal agents are the enteric pathogens; *Escherichia* sp., *Shigella* sp., *Vibrio* sp., *Salmonella* sp., *Staphylococcus* sp., etc. So, probiotics with therapeutic use has become a fast alternative to reduce the antibiotic consumption and subsequent enteric infection **(Ballal and Shivananda, 2002)**. As *Lactobacillus* sp (Lactic acid bacteria; LAB) are one of the group of intestinal microflora as a healthy partner and symbionts causing various health benefits, researchers have paid attention to these microbes by using it in fermented foods as probiotics.

Lactic acid bacteria (LAB) is a group of acid producing bacteria used in the food industry and making starter culture for dairy products **(Gharaei-Fathabad and Eslamifer, 2011)**. The proper selection and balance for starter culture is critical for the manufacture of fermented products of desirable texture and flavor. Members of this group include gram-stain-positive, non-spore forming, cocci or rods with catalase negative fastidious organism belonging to family *Lactobacillaceae* of phylum *Firmicutes* **(Felis and Dellagio, 2015)**. These organisms have been considered as "Generally Recognized as Safe" (GRAS) due to their positive impacts on health in the form of probiotics **(Forouhandeh *et al*., 2010)**. They are known to produce antagonistic substances such as hydrogen peroxide, carbon dioxide and bacteriocin other than organic acids (lactate and acetate) against food borne pathogens and spoilage microorganism and hence extend the shelf life and safety of the products **(Durne *et al*., 2001; Chowdhury *et al*., 2012)**. Bacteriocin of LAB is most promising extracellular bactericidal product (mostly proteins) that is secreted by the LAB **(Tagg *et al*., 1976; Holzapfel *et al*., 1995)**. Bacteriocins produced by LAB are considered as safer natural or bio-preservatives, as they are degraded by the proteases in gastrointestinal tract unlike traditional antibiotics. They can also reduce the use of chemical preservatives in foods **(Cleveland *et al*., 2001)**. On the basis of the protein structure, bacteriocin constitutes a heterogeneous group of small peptides of high molecular weight protein or protein complexes. The inhibition spectrum of bacteriocin produced by LAB towards gram positive bacteria varies widely but is most confined to closely related species of the producing bacteria and gram negative members **(Puniya *et al*., 2012)**. Animal studies and human clinical research have shown lactobacilli being able to accelerate recovery from a range of intestinal conditions, and preventing infection **(Mobarez *et al*., 2008)**. The foremost mechanism of antagonistic property of LAB is attributed to decrease pH by producing acid and production of other primary and secondary antimicrobial metabolites as a result of fermentation process. Most of the probiotics lactobacilli in human foods are supplied in highly concentrated forms containing more than 10^{10} CFU/g **(Parada *et al*., 2007)**. There are different ways by which lactobacilli may produce probiotic effects such as; acidifying the intestine, attaching to the intestinal lining, producing various anti-microbial substances, competing with pathogens for nutrients, modulating the host immune system and alleviating lactose intolerance etc **(Asha and Gayatri, 2012)**. At the time of writing, there are 214 species of *Lactobacillus* with quite a broad range of characteristics, but only a few have been used in probiotics products, including: *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus crispatus*, *Lactobacillus delbrueckii*, *Lactobacillus johnsonii*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus* and *Lactobacillus salivarius* (www.bacterio.net/LPSN/Lactobacillus).

Considering all the research need and future implications of the potential *Lactobacillus sp.* in different fermented food industries and human health sector, the present study was conducted to isolate and characterize indigenous *Lactobacillus sp.* from the curd samples available in the local market of Bhubaneswar, Odisha, India. Subsequently their growth behavior, physiological characteristics and in-vitro antagonistic activity was assessed against some enteric pathogens. The best potential strains showing maximum activity were selected for identification through 16S rRNA gene sequencing and its evolutionary relationship with other *Lactobacillus sp.* members.

MATERIALS AND METHODS

Sample collection

Two curd samples of different variety available in the local market of Bhubaneswar, Odisha, India were collected in a sterile polypropylene vials after flushing the vials twice with the same samples inside laminar air flow in the laboratory. The pH of the samples was checked with help of pH electrode by diluting it with sterile distilled water in a ratio of 1:2. The samples were stored at 4°C for further processing and isolation of *Lactobacillus* sp.

Isolation, identification and characterization of *Lactobacillus* species

Lactic acid bacterial strains were isolated from the curd samples using deMan Rogosa Sharpe (MRS) plates acidified with 1N HCl to adjust the pH from 6.7 to 4.5 to inhibit the growth of other fastidious organisms. 1 gram of curd sample suspended in 9 mL sterile normal saline solution, shaken at 150 rpm for 15 min, diluted serially by 10 fold dilution and 0.1 mL aliquots of appropriate dilutions were plated on MRS plates, and incubated anaerobically in anaerobic gas jar at 37ºC for 3-5 days. Based on colony morphology and pigmentation, individual colonies were picked up and purified by repeated sub culturing on the same medium. The cultured isolates were characterized on the basis of gram staining, endospore formation ability, and biochemical characteristics as described in Bergey's Manual of Determinative Bacteriology **(Holt *et al*., 1994)**. Biochemical characterization was performed in terms of assimilation of different sugars (arabinose, cellobiose, esculin, D-fructose, galactose, maltose, mannose, melibiose, raffinose, rhamnose, sorbitol, sucrose, trehalose and xylulose), Production of H_2S, gas and enzymes (such as amylase, urease, catalase, and nitrate reductase), assimilation of sulphate and iron and growth in anaerobic condition.

Tolerance to different sodium chloride (NaCl) concentrations and pH

For determination of NaCl tolerance, all the isolates were grown in MRS broth amended with different concentrations of NaCl (1-8% [w/v]). 1% (v/v) of the freshly grown overnight culture of the isolates were inoculated into the test tube containing MRS broth medium and incubated at 30ºC for 48 h. Growth was checked using a UV-VIS spectrophotometer (Cary 50, Varian, USA) by measuring optical density (OD) at 600 nm. Optimum pH for the growth of the isolates was determined by inoculating 1% (v/v) of the overnight culture of the isolates into MRS broth with varying pH ranging from 3.0 to 11.0. The acidic range of the pH was set by adding 1N HCl and alkaline range was achieved by adding 1N NaOH and measuring pH with pH probe electrode. The inoculated broths were then incubated at 30°C for 48 h. Growth of the bacterial isolates was measured as mentioned above. Growth with optical density <0.5 termed as no growth, >0.5 as moderate growth and >1.0 as luxuriant growth.

Determination of antibiotic susceptibility of the isolates

Antibiotic susceptibility test was carried out by Kirby Bauer disc diffusion method on Mueller Hinton agar **(Bauer et al., 1966)**. The antibiotics(each 30 µg) discs (Hi-Media, India); amikacin, amoxicillin, amphotericin-B, ampicilin, azithromycin, bacitracin, cefactam, cefepime, cefixime, cephadroxil, cephataxime, chloramphenicol, erythromycin, gatifloxacin, gentamicin, kanamycin, methicillin, neomycin, norfloxacin, penicillin-G, polymixin-B, rifampicin, streptomycin, tetracycline and vancomycin were placed on lawn culture of bacterial strains on Muller-Hilton agar medium and incubated at 30ºC for 24h. The zone of inhibition around the well confirmed strain's inability to grow in presence of antibiotics (sensitive or susceptible) and no zone of inhibition indicated resistance against those antibiotics. All experiments were performed in triplicates.

Production of crude bacteriocin

The isolates from the curd sample were tested for their ability to produce bacteriocin. The isolates maintained in MRS agar were inoculated in to MRS broth and incubated at 37°C for 48 hours. Cells were separated by centrifugation at 5000 rpm for 10 minutes. The cell free supernatant was passed through 0.45 µm membrane filter and the resultant filtrate containing bacteriocin was evaluated for antimicrobial activity.

Test for antagonistic effect of the isolated strains/extract on enteric pathogens

All the isolated LAB strains and their bacteriocin extract were subjected to screening for antagonistic activity against indicator pathogens by agar well diffusion method and spot-on-the-lawn technique. *E. faecalis*, *S. boydii*, *E. coli* and *S. typhi* pathogenic strains were used as indicator organisms. The culture of the indicator strains were prepared by pouring 1 mL of the inoculum onto MHA plates to completely cover the surface of the agar. Six mm diameter wells were produced by punching into the agar using sterilized well puncher. 20µL of each LAB strain inoculum ($1 \times 10^{3-4}$ CFU/mL) was placed into each well carefully with sterile pipette. In case of bacteriocin extract, 20 µL of the aliquot was poured into each well. The zone of inhibition was visualized around the wells were recorded after incubating the plates for 48 h at 37°C. In the spot-on-the-lawn technique, 1 mL of each LAB strain inoculum added to 15 mL of the MRS medium, plated and incubated at 37°C for 24 h. After incubation, plates were overlaid with 10 mL of MHA semi-solid agar (0.8g/100mL) and inoculated with 10^{4-5} CFU/mL of indicator organisms. The plates were then incubated at 37ºC for 24 h for visualization of inhibition zone around the spots. Spot showing clear zone was considered a positive antagonistic effect. The strains with best antagonistic effect in all the conditions were selected for identification by 16S rRNA gene sequencing and phylogenetic analysis.

Amplification of 16S rRNA gene and Phylogenetic analysis

Genomic DNA of the selected strains was isolated from 24h grown culture in MRS broth by using phenol/chloroform extraction followed by purification with Isopropanol. Partial sequence of the 16S rRNA gene was amplified from the genomic DNA by PCR thermal cycler by using universal eubacterial primers: 27F/1492R with an initial denaturation step at 95°C for 10 min, followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2 min and a final extension at 72°C for 5 min. Gel slices containing the amplicons DNA were cut under a UV Trans-illuminator and transferred into an eppendorf tube for gel extraction. The PCR amplicons were gel purified using Qiaquick PCR purification kit (Qiagen, Germany) according to manufacturer's instructions and sequenced. The obtained sequence chromatogram was compared with sequences available within the NCBI database (http://www.ncbi.nih.gov) by BLASTn search using FASTA sequence. Basing on the maximum sequence homology, sequences of the most closely related species were retrieved from RDP database and all of the sequences were aligned by using ClustalW (http://www.ebi.ac.uk/clustalw/). Pair wise evolutionary distances were calculated using the algorithm of Jukes and Cantor and the phylogenetic dendrogram were constructed using the neighbor–joining (NJ) method with bootstrap analysis based on 1000 replications using the program MEGA (version 5.1). For evaluation of robust phylogenetic relationship, the obtained nucleotide sequences were also checked for its identification through EzTaxon server (http://www.ezbiocloud.net/eztaxon) with bacterial type strains of validly described taxa, where the ambiguous bases were removed from the assembled sequences and searched against the cultured species for maximum sequence similarity (% identity).

Growth behavior study of the selected bacterial strains

In this study, two of the selected isolated strains; OC3 and KC2 were chosen for study of growth behavior and kinetics. Pure cultures of the each bacterial strain (single colony from the MRS agar plate) were transferred to 5 ml of the MRS broth for preparation of starter inoculum by incubating at 37°C for 24 h. 1% (v/v) of freshly grown culture of each strain (OD 570 nm~ 0.5-0.6) was transferred to sterile 250 mL Erlenmeyer flask containing 100 mL of the MRS broth. One flask with *Escherichia coli* (a laboratory strain) was taken as internal control, while medium without any inoculum was used as blank. All the flasks were incubated in a shaker incubator at 37°C for 24 h. Growth was checked by measuring optical density of the culture with the spectrophotometer at 570 nm over the period of time in interval. Specific growth rate (µ) of each strain was calculated by following equation:

$$\frac{\text{Log Nt} - \text{Log No}}{t1 - t0} = \frac{\mu}{2.303}$$

Where, Nt = Absorbance at initial log phase of the growth
N_0 = Absorbance at final log phase of the growth
t_1- t_0 = time difference from Nt to N_0

RESULTS AND DISCUSSION

Fermented milk products have become a potential carrier of antimicrobial compounds and serve as suitable alternative of antibiotic consumption. Mostly dairy products with probiotics nature has become as an example of classical food with nutritive value **(Kyriacou *et al*., 2008)**. Recent research has been focused on exploring suitable probiotics candidate contained within fermented dairy products

such as curd, yoghurt etc for their antagonistic property against enteric pathogens. The purpose of this study was to isolate and characterize potential probiotic *Lactobacillus* species from curd samples available in the local market of Bhubaneswar, Odisha, India and to explore their antagonistic activity against enteric pathogens. Further, to confirm their taxonomic affiliation, 16S rRNA gene sequence was analyzed and phylogenetic relationship was inferred.

Sample collection and analysis

Two curd samples from the local market was procured and designated as OC and KC. Both the samples were found to be acidic in nature with average pH value of 4.5-5.5. Samples were further analyzed for isolation of *Lactobacillus* species.

Isolation, identification and characterization of *Lactobacillus* species

Isolation of *Lactobacillus* strains has been accomplished by using *Lactobacillus* selective medium MRS agar. A total of 10 bacterial isolates were isolated from both the curd samples based on their colony characteristics and pigmentation on MRS plates. All the isolates were designated as OC1 to OC5 (5 colonies from sample OC) and KC1 to KC5 (5 colonies from sample KC). The morphological and biochemical characterization of the isolates were carried out and the results are mentioned in the table1. All the isolates were found to be gram positive (+ve) bacilli without ability to formation of endospore. Catalase, urease, amylase, production of H_2S, nitrate reduction and motility tests were found to be negative for all the isolates. Among various carbohydrate used for assimilation, all the isolates assimilated arabinose, cellobiose, fructose, lactose, mellobiose, maltose, mannose, sorbitol, sucrose and trehalose. Out of 10, nine isolates showed positive response to galactose and rhamnose. Only two of the isolates could able to use esculin and raffinose (Table 1, Fig 1). Interestingly, lactose was found to be used by all, as it is the principal sugar available in the milk and milk products. All the isolates also showed same anaerobic growth pattern. Hence, from the biochemical properties, it was confirmed that all the isolates showed determinative properties of members of *Lactobacillus* sp. As probiotics bacteria mostly consumed by human being directly by oral administration, they must be tolerant to varying pH (acidic in the stomach and alkaline in the intestine) and different salt (NaCl) concentration **(Davidson, 2003)**. So, pH tolerance and temperature tolerance profile were studied for all the isolates (Fig 2). Among all, only two isolates (OC3 and KC2) showed tolerance from pH 4 to 9. Almost all showed luxuriant growth in pH 6 to 8 showing their near neutral growth behavior. OC3 and KC2 were found to be most resistant towards acid and basic pH showing their versatility to resist all pH condition of gastro-intestinal tract. In the study of salt tolerance profile, all showed almost similar pattern of growth where, 5% NaCl was found to be the maximum concentration above which growth was inhibited. OC2, OC3, KC1 and KC4 were the most potent in terms of using NaCl during their growth.

Table 1 Differential phenotypic characteristics of isolated bacterial strains from curd samples

Properties	Bacterial strains									
	OC1	OC2	OC3	OC4	OC5	KC1	KC2	KC3	KC4	KC5
Gram reaction	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Shape	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli
pH (opt)	5.5-6.5	5.5-6.5	5.5-6.5	5.5-6.5	5.5-6.5	5.5-6.5	5.5-6.5	5.5-6.5	5.5-6.5	5.5-6.5
Temp(° C)	30-35	30-35	30-35	30-35	30-35	30-35	30-35	30-35	30-35	30-35
Endospore	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Catalase	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
H_2S production	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Nitrate reduction	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Starch hydrolysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Urease	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Mannitol motility	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Strains; OC1 to OC5 (from sample 1), KC1 to KC5 (from sample 2), pH (opt); pH optimum, Temp (° C); Temperature in degree centigrade, +ve; Positive, -ve; Negative.

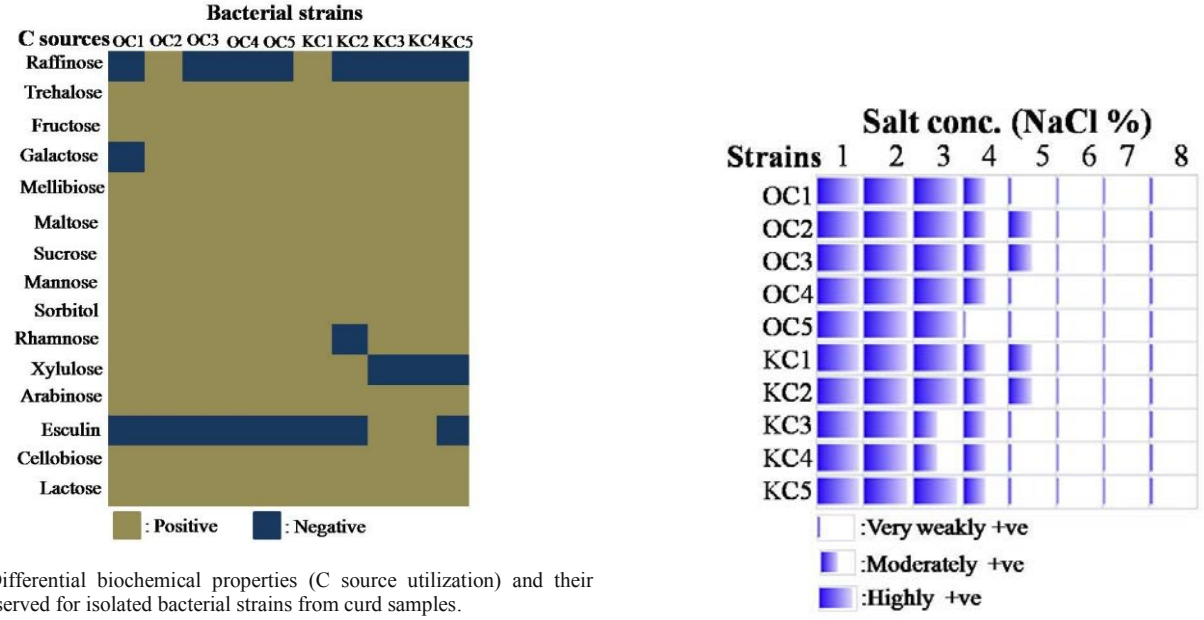

Figure 1 Differential biochemical properties (C source utilization) and their response observed for isolated bacterial strains from curd samples.

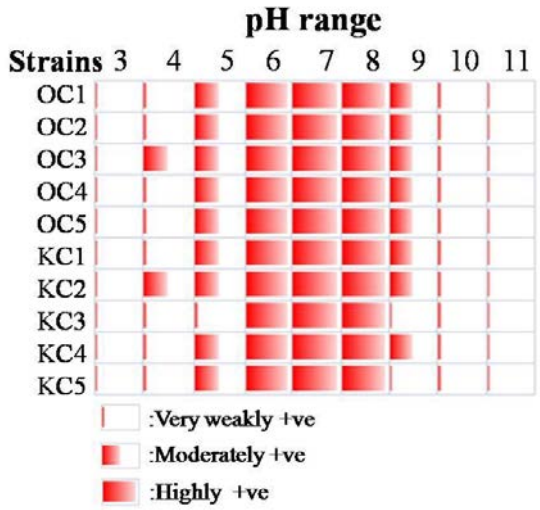

Figure 2 Physiological profile of the isolated strains from the curd samples; pH tolerance and salt (NaCl) tolerance.

Determination of antibiotic susceptibility of the isolates

Generally, antimicrobials have been used as a primary intervention for inactivation or of pathogenic microbes in foods **(Salminen, 1998)**. The optimization of the use of probiotic LAB for the gastrointestinal disorders requires the knowledge of their antibiotic resistance for the development of antibiotic therapy **(Pan *et al*., 2009)**. Antibiotic resistance profile of all the isolates was studied and presented in table 2. All the isolates showed resistance to almost all the antibiotics tested. Isolates from two curd samples (OC and KC), five isolates from OC were susceptible to ampicilin, cefataxime and polymixin-B, while only two isolates; OC2 and OC5 showed sensitivity towards amphotericin-B. Among five isolates from KC sample, susceptibility towards polymixin-B was observed for all, where as two isolates; KC2 and KC5 showed susceptibility to amphotericin-B. Study of the antibiosis profile of the probiotics strains is one of the most promising characteristics to be used in food and food products for getting health benefits **(Ahmed, 2013)**. Our result also showed a good agreement of antibiotic resistance ability of the strains with the previous report. Because, tolerance towards antibiotics can lead to persistency of strains inside intestinal tract but it could transfer resistance to other bacterial members through transfer of resistance gene **(Mathur and Singh, 2005)**.

Table 2 Antibiotic susceptibility profile of the isolated strains showing resistance to different antibiotics

Antibiotics	Bacterial strains									
	OC1	OC2	OC3	OC4	OC5	KC1	KC2	KC3	KC4	KC5
Erythromycin	–	–	–	–	–	–	–	–	–	–
Amikacin	–	–	–	–	–	–	–	–	–	–
Polymyxin-B	+	+	+	+	+	+	+	+	+	+
Gentamicin	–	–	–	–	–	–	–	–	–	–
Rifampicin	–	–	–	–	–	–	–	–	–	–
Neomycin	–	–	–	–	–	–	–	–	–	–
Gatifloxacin	–	–	–	–	–	–	–	–	–	–
Streptomycin	–	–	–	–	–	–	–	–	–	–
Amoxycillin	–	–	–	–	–	–	–	–	–	–
Amphotericin-B	–	+	–	–	+	–	–	+	–	+
Methicillin	–	–	–	–	–	–	–	–	–	–
Kanamycin	–	–	–	–	–	–	–	–	–	–
Norfloxacin	–	–	–	–	–	–	–	–	–	–
Cefactor	–	–	–	–	–	–	–	–	–	–
Ampicillin	+	+	+	+	+	–	–	–	–	–
Penicillin-G	–	–	–	–	–	–	–	–	–	–
Vancomycin	–	–	–	–	–	–	–	–	–	–
Azithromycin	–	–	–	–	–	–	–	–	–	–
Chloramphenicol	–	–	–	–	–	–	–	–	–	–
Cefixime	–	–	–	–	–	–	–	–	–	–
Cefepime	–	–	–	–	–	–	–	–	–	–
Bacitracin	–	–	–	–	–	–	–	–	–	–
Tetracyclin	–	–	–	–	–	–	–	–	–	–
Cephadroxil	–	–	–	–	–	–	–	–	–	–
Cephataxime	+	+	+	+	+	–	–	–	–	–

Strains; OC1 to OC5 (from sample 1), KC1 to KC5 (from sample 2), +; Susceptible, **-;** Resistant

Test for antagonistic effect of the isolated strains/extract on enteric pathogens

Almost all the isolates showed inhibition against all the test pathogens (Table 3). In the spot inoculation method, highest antagonistic activity was achieved against *E. coli* where seven isolates (OC1, OC2, OC3, KC1, KC2, KC4 and KC5) showed positive response. OC1, OC3, OC5, KC1, KC2 and KC4 (six) inhibited the growth of *S. typhi* and while OC2, OC4, KC1 and KC5 against *S. boydii* and OC1, OC3, OC5 and KC2 against *E. faecalis*. Among all the isolates, OC1, OC2, KC1 and KC2 demonstrated highest activity after 48 h of incubation. Well diffusion method showed a better result of antagonism compared to spot inoculation, where all the isolates showed inhibition against almost all the pathogen tested. 70% of them showed highest activity against *E.coli* and *S. typhi* followed by *S. boydii* and *E. faecalis*. The strain OC5 showed inhibitory action

against all the enteric pathogens and found to be the best candidate (Table 3). But the strains OC2, OC3 (with moderate to high antagonistic activity), KC2 (with moderate antagonistic) were consistent among all according to both the spot and well diffusion methods. From the bacteriocin extract assay, it was observed that same strains; OC3 and KC2 found to be with moderate to highest antagonistic activity after 24 as well as 48 hours of incubation. However the well diffusion method of whole cell application showed a slightly better result compared to bacteriocin extract. Previous study also confirmed the similar result where the test *Lactobacillus* sp. members showed highest activity against *E.coli* followed by *S. typhi*, *S. boydii* and *E. faecalis* **(Gharaei-Fathabad and Eslamifer, 2011)**. Our result also confirmed the similar antimicrobial property. *Lactobacillus* sp. members isolated from dairy and fermented dairy products also showed same type of inhibitory result against the enteropathogenic *E. coli* and *S. typhi* **(Osuntoki *et al*., 2008)**. The antagonistic attributes could be due to production of organic acid (lactic acid, acetic acid or other acid or other metabolites) and subsequent decrease in pH causing inhibition of pathogen's growth. As the test strains was found to be most closely related to *L. plantarum* and *L. brevis*, the effects on enteric pathogens is quite obvious. As previous study **(Morgan *et al*., 2009)** confirmed the antagonistic potential of *L. plantarum* strains isolated from a probiotics food.

Table 3 Antagonistic effect of the indigenous bacterial strains and their bacteriocin extract on enteric pathogens

Pathogenic strains	**Bacterial strains (Spot inoculation method)**									
	OC1	OC2	OC3	OC4	OC5	KC1	KC2	KC3	KC4	KC5
Enterococcus fecalis	+	-	++	-	+	-	++	-	-	-
Shigella boydii	-	++	-	+	-	+	-	-	-	+
Escherichia coli	+	+	-	+	-	+	++	-	+	+
Salmonella typhi	+	-	++	-	+	+	++	-	+	-
Pathogenic strains	**Bacterial strains (Well diffusion method)**									
	OC1	OC2	OC3	OC4	OC5	KC1	KC2	KC3	KC4	KC5
Enterococcus fecalis	+	-	++	-	+	+	++	-	+	-
Shigella boydii	-	+	-	+	+	+	-	+	-	+
Escherichia coli	+	-	++	+	+	+	-	+	-	+
Salmonella typhi	+	+	++	-	+	-	++	-	+	+
Pathogenic strains	**Bacteriocin extract from bacterial strains (Well diffusion method)**									
	OC1	OC2	OC3	OC4	OC5	KC1	KC2	KC3	KC4	KC5
Enterococcus fecalis	+	+	++	-	+	-	+	-	+	-
Shigella boydii	-	-	-	+	-	+	+	+	-	+
Escherichia coli	+	-	++	-	-	+	-	+	-	-
Salmonella typhi	+	-	++	+	+	-	+	-	+	-

Strains; OC1 to OC5 (from sample 1), KC1 to KC5 (from sample 2), **+;** Inhibition, **++;** Moderate inhibition, **-;** No inhibition

Amplification of 16S rRNA gene and Phylogenetic analysis

16S ribosomal ribonucleic acid (rRNA) analysis and sequencing is one of the measure current methodologies, on which classification of LAB mainly rely on **(Rauta *et al*., 2013; Drancourt *et al*., 2000)**. Among all the bacterial isolates, OC3 and KC2 showed positive response in all the studied properties and hence were selected for identification and phylogenetic analysis by partial 16S rRNA gene sequence based method. The genomic species level identification was defined as a 16S rDNA sequence similarity above 99% with the query sequence **(Murray *et al*., 2009)**. A total of 1370 nucleotide stretch of 16S rRNA gene for strain KC2 and 1404 nucleotide stretch for OC3 were obtained after the sequencing. Based on these partial sequences, BLASTn search was performed and KC2 showed 99% (with 100% query coverage) identity match with *Lactobacillus plantarum* strains (accession: KP345895, KP230423, KM982988 and KP178090). While, OC3 showed 99% (with 99% query coverage) similarity with *Lactobacillus brevis* strain (accession: AB626062, AB024299, KP889228 and LC062897). All the strains showed maximum sequence homology with *Lactobacillus* sp. members. Hence, the type strains of genus *Lactobacillus* were retrieved from RDP database and aligned with the sequence of both the strains by both pairwise and multiple alignment and phylogenetic dendrogram tree was constructed by neighbor joining (NJ) algorithm of Jukes-Cantor by MEGA 5.1. Based on phylogeny, the tree showed close taxonomic relatedness of the strain KC2 with the clade containing the type strains including *L. xiangfangensis* 3.1.1. (Isolated from Chinese pickle), *L. fabifermentans* LMG 24284 (cocoa fermentation), *L. paraplantarum* DSM 10667 (fermented beer), *L. plantarum* DK022 and *L. plantarum* NRRLB-14768 (Korean kimchi) having 100% bootstrap value. OC3 was cladded with *L. brevis* ATCC 14869 type strain with 100% bootstrap value showing close taxonomic affiliation to *Lactobacillus* sp (Figure 3). The same tree was also inferred from maximum-likelihood method for confirmation of robustness of the tree (data not shown). Interestingly it was observed that, the test strains (KC2 and OC3) showed close taxonomic relatedness with the *Lactobacillus* sp isolated from different fermented foods and confirmed its relationship, as the strains have also been isolated from fermented milk product (curd). The present results corroborates with the previous findings **(Jagadeswari *et al*., 2010; Shantnya *et al*., 2011)** demonstrating isolation of *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus brevis* and *Lactobacillus acidophilus* from raw goat milk.

Genebank accession number

The partial 16S rRNA gene sequences of the strains KC2 and OC3 were isolated and deposited in the GeneBank (http://www.ncbi.nlm.nih.gov/genbank/) and under the following accession number KT380050 and KT380051.

Growth behavior study of the selected bacterial strains

The strain KC2 and OC3 were selected for study of growth behavior and kinetics as they performed well in all the experiments. Both the strains showed almost similar growth pattern showing very less (around 1 hr) lag phase (Figure 4). Multiplication of cells in logarithmic phase started from 2 hrs and persisted up to 12 hrs. Although, both the strains showed similar multiplication rate at initial log phase but after 6 hrs, showed slight divergence in growth rate (Figure 4). The end of logarithmic phase was noticed with the final OD of around 1.6. Growth rate (μ) was calculated and found to 0.42 h^{-1} for KC2 and 0.32 h^{-1} for OC3 showing high growth rate of KC2 compared to OC3. But, interestingly for the strain OC3, stationary phase persisted for a long time while, death phase was noticed for the strain KC2 followed by short stationary phase. Compared to test strains, *E.coli* (internal control) showed a slow growth rate (μ = 0.2 h^{-1}), with a long lag phase of 5-6 hrs. In log phase, highest OD achieved was found to be 0.8, just half of the OD obtained for test strains. While blank showed no significant increase in turbidity and hence OD, confirming its sterility. The growth behavior noticed in this study coincided with the growth characteristics of *Lactobacillus* sp. obtained in previous study **(Asha and Gayatri, 2012; Brizuela *et al*., 2001)** confirming the growth rate of *Lactobacillus* sp. to be around 0.5 h^{-1}. Since *Lactobacillus* species are involved in a large number of spontaneous food fermentation and processing and also inevitable for the human health, Use of such organism as probiotics is a good choice for human health. In the present study, two *Lactobacillus* sp. (OC3 and KC2) affiliated to *L. paraplantarum* and *L. brevis* showed broad antibiotic resistance with antimicrobial activity against pathogenic microorganisms, tolerance to wide pH, salt (NaCl) concentrations were obtained. These strains can be used as starter inoculum for fermented dairy products and as potential probiotics agents.

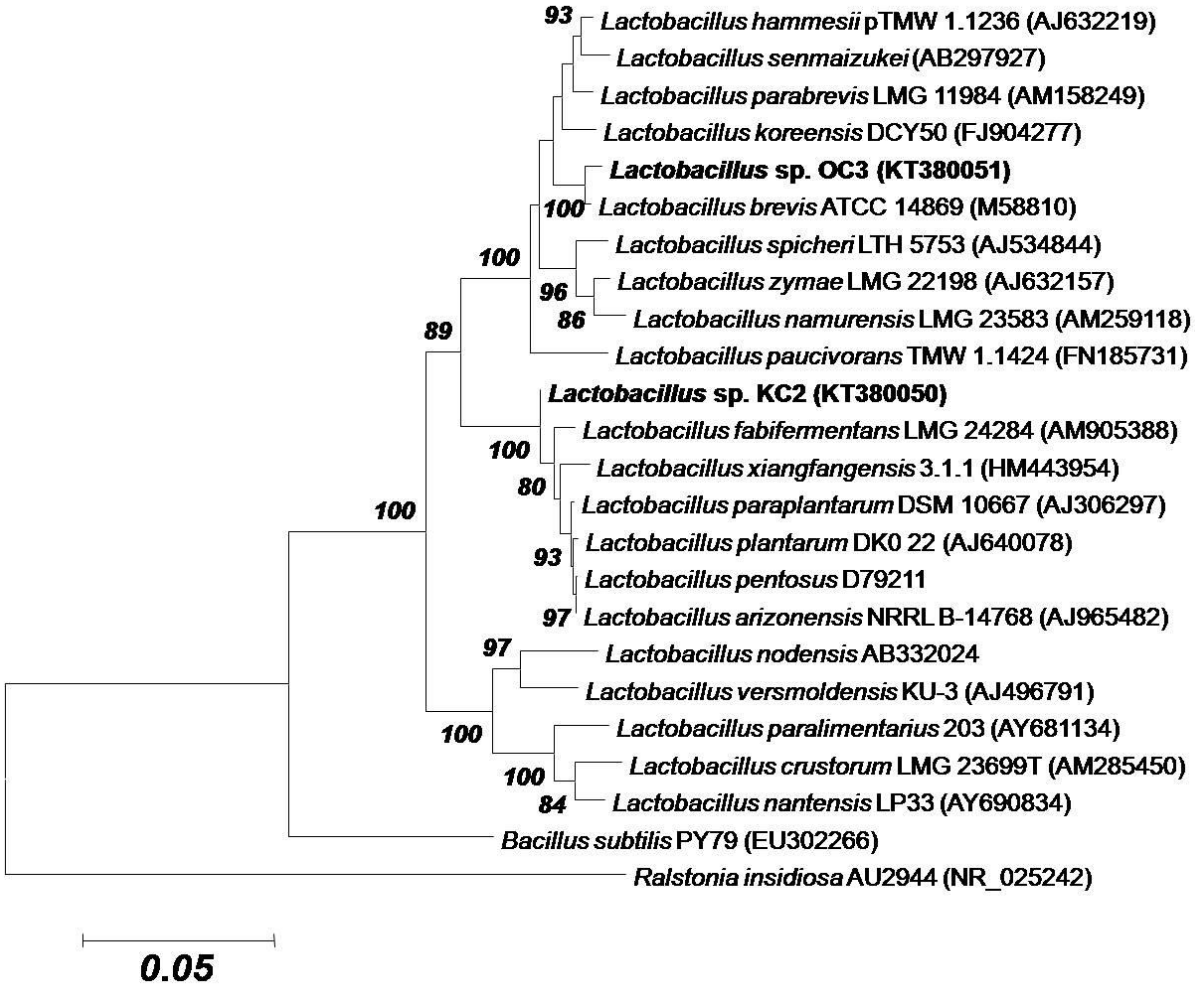

Figure 3 Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence of two selected strains; OC3 and KC2 with type strains of closely related species within the genus *Lactobacillus*. The significance of each branch is indicated by a bootstrap value calculated for 1000 replications. Represented type strains of the *Lactobacillus* sp. and outgroup is presented along with the test strains. Bootstraps of above 70% are shown at each branch points. GenBank accession number of 16S rRNA gene sequences of each strain is in parentheses. Bar 0.05 indicates 5% nucleotide substitution.

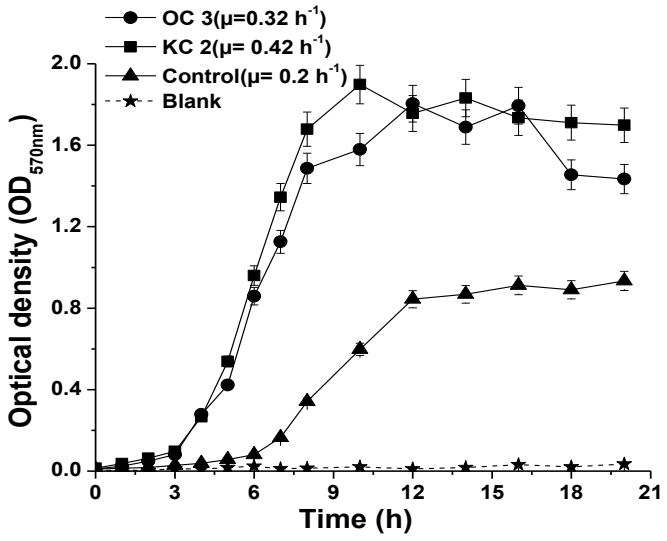

Fig 4 Growth profile of the bacterial strains; OC3 and KC2 in MRS broth medium, where *E. coli* was taken as internal control. Specific growth rate (μ) has been calculated and mentioned in parentheses. Error bar indicates standard deviation, where n= 3.

CONFLICT OF INTEREST

No conflict of interest declared.

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CHARACTERIZATION OF POTENT EXOPOLYSACCHARIDE PRODUCING BACTERIA ISOLATED FROM FRUIT PULP AND POTATO PEELS AND ENHANCEMENT IN THEIR EXOPOLYSACCHARIDE PRODUCTION POTENTIAL

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ABSTRACT

Exopolysaccharides (EPS) are environment friendly natural polymers secreted by microorganisms in the surrounding medium. Due to the presence of unique structural composition, EPS shows diverse applications such as in food formulations, pharmaceutical, cosmetics industry, etc. In the present investigation for the isolation of EPS producing bacteria, 30 samples comprising 14 fresh fruits, 14 spoiled fruits, 1fresh potato peels and 1spoiled potato peels were inoculated on 4 different solid media containing sucrose, glucose and galactose as carbohydrate substrate and MRS agar (MRSA). The bacteria producing higher exopolysaccharide were screened among bacteria isolated from fresh and spoiled fruits and potato peels. Total 105 EPS producing colonies were obtained, out of which 17 isolates, which showed viscosity more than 0.1060m.Pa.S^{-1} were selected. Based on 16S rRNA gene sequence analysis, 17 isolates, which represents 8 genera, out of this 40% isolates belong to genera *Bacillus*. Among the carbohydrate studied sucrose proved to be the choice of the isolates for EPS production as compared to other sugars. When SYE and EPS media were compared, EPS medium was found to be the best except for the isolate SR17. The selection of EPS medium as medium of choice, sucrose as source of sugar and its 5% concentration in the medium enhanced EPS production as high as 440% higher as compared to SYE medium.

Keywords: Exopolysaccharide, isolation, identification, fresh and spoiled fruits, potato peels, viscosity

INTRODUCTION

Exopolysaccharides (EPS) are key components of biofilm, which determines physico-chemical, and biological properties of biofilm formation. The exopolysaccharide play an important role in allowing microbes to live continuously at high cell densities in a stable mixed population of biofilm communities. Bacteria that produce exopolysaccharides have been identified from a variety of ecological niches and it is apparent that precise role played by exopolysaccharides is dependent on the natural environment, from which they are isolated. Ability to produce exopolysaccharides is a direct and logical response to selective pressures in that natural environment (**Weiner, 1997**).

EPS are used in food, textile, detergents, beverages, pharmaceutical (**Nwodo *et al*., 2012**), biotechnology, agricultural, paper, paint, cosmetic, medical and petroleum industries (**Quesada *et al*., 1993**) drug delivery (**Sosnik, 2014**), cancer therapy (**Zhang *et al*., 2013**) and in the formulation of the culture media due to their unique structure and physical properties. Some of these applications include their use as emulsifiers, stabilizers, binders, gelling agents, coagulants, lubricants, film formers, thickening and suspending agents (**Sutherland, 1998**). Due to their bioactive role and their extensive range of applications increasing attention is being paid to the production of these biomolecules. A new approach to encounter EPS with novel properties might entail investigating different environments. Isolation of bacteria from fresh vegetables and fruits, ready to eat salads (**Trias *et al*., 2008a**), citrus fruits waste (**Marína *et al*., 2007**), vegetable salads (**Wright *et al*., 1976**), processed vegetables (**Franzetti *et al*., 2005**) dried fruits (**Askari *et al*., 2012**) have been done but with the importance of these bacteria in food spoilage (**Nguz *et al*., 2005**), pathogenicity (**Palacio *et al*., 2012**), antibacterial activity (**Askari *et al*., 2012**) and production of organic acids (**Aslim *et al*., 2005; Trias *et al*., 2008b; Mridul and Preethi, 2014**). To the best of our knowledge, no data are available for the isolation of EPS producers from potato peels and fruit pulp. Thus, in this context attempts were done to isolate EPS producing bacteria from potato peels and fruits. Work was also carried out to enhance their EPS production potential.

MATERIAL AND METHODS

Screening and isolation of exopolysaccharide producing bacteria

From the local market 14 types of fresh fruits and potato peels were procured and used for isolation of EPS producing bacteria. One gram pieces of fresh fruit and potato peels were homogenized in 10 ml sterile peptone broth and incubated at 28±2 °C for 24 h. After incubation, 10 folds serially diluted suspensions were prepared and 0.1 ml aliquots were spread on yeast extract (YE) agar plates containing glucose, galactose or sucrose as carbon source. Fruits were stored at 28±2 °C and were allowed to spoil and after every 15 days, isolation was performed up to two months as mentioned for fresh samples. Plates were incubated for 48 h at 28±2 °C. Mucoid colonies were screened and re-streaked on another agar plate with the same composition to obtain a pure culture. de Man, Rugosa and Sharp (MRS) medium **(Atlas and Parks, 1997)** was also used for isolation. Isolated cultures were characterized on the basis of colony morphology, microscopic observations and routine biochemical tests. The identification work was done according to the methods described in Bergey's Manual of Systematic Bacteriology **(Brenner *et al*., 2005; Vos *et al*., 2009)**. Selected cultures were further identified by 16S rRNA partial gene sequence analysis.

Ehancement in EPS production

EPS production was carried out in 250 ml Erlenmeyer flasks containing 100 ml of medium. The base medium contains (g/L): yeast extract 1.0; $MgSO_4$ 0.5; KH_2PO_4 1.0 and glucose/galactose/sucrose/mannitol 30.0 as a source of carbohydrate. The pH of all four media was adjusted to 7.0. All media constituents were sterilized at 121°C for 20 min except carbohydrates. All carbohydrates were filter sterilized and added to the sterile base medium under aseptic condition. All the test flasks were inoculated with actively growing 15 hours old 10% v/v culture, having cell count of 10^7 cells/ml. The flasks were incubated on a rotary shaker at 28±2 °C for 5 days. Viscosity and production were checked after every 24 h. A comparative study for production of EPS was also performed using EPS medium (g/L): caseine hydrolysate 15.0, sodium acetate 12.0, K_2HPO_4 10.0, yeast extract 5.0, sodium chloride 2.5, L-cystine 0.5

and sucrose/glucose/galactose 50.0, pH 7.2 **(Atlas and Parks, 1997)**.Influence of carbohydrate on EPS production was studied by supplementing medium with 30g/L glucose/galactose/sucrose/mannitol. Further concentration of sucrose in the medium was also optimized by providing 30,50 and 70g/L sucrose.

Recovery of EPS

Cells were harvested from the respective broth medium by centrifugation at 10,000 g for 10 min. After centrifugation, three volumes of chilled acetone was added into the supernatant and stored overnight at 4 °C. Precipitated material was collected by centrifugation (10 min at 10,000 g) and the pellets were dried at 65 °C till constant weight. Dry weight was measured from all the dried pellets **(Razack *et al*., 2013)**.

RESULTS AND DISCUSSION

All the colonies on 4 media were observed, based on difference in colony morphology 105 bacterial isolates were selected as EPS producers as they showed slimy gummy colony morphology (Table 1). Out of 105 isolates, 68 bacterial isolates were from fresh samples and 37 from spoiled samples. Medium with sucrose resulted in growth of nearly 50% of the total isolates. As compared to spoiled fruits or potato peels, fresh samples showed more variety of EPS producers except watermelon sample. In case of sucrose containing medium 6 types of colony reappeared in spoiled samples, whereas in glucose containing medium 3 samples showed reappearance of single isolate. In case of galactose and MRSA none of the samples showed the presence of isolates isolated from fresh sample. During the course of two months storage out of 68 isolates which were isolated from fresh samples 14% colony reappeared in spoiled samples. A number of bacterial isolates decreased during storage of samples, due to drying of samples as well as the antagonists effect of the survived organisms.

Table 1 Isolates and source of isolation

Source	Number of morphologically different colonies picked from yeast extract agar with different cabohydrates and MRS agar (MRSA)							
	Sucrose		Glucose		Galactose		MRSA	
	Fresh	Spoiled	Fresh	Spoiled	Fresh	Spoiled	Fresh	Spoiled
Potato peels	3(1)	1	3	1	2	1	-	-
Orange	4(2)	1	2	1	1	-	-	-
Apple	1	1	2(1)	1	2	-	-	-
Chikoo	3(1)	1	2	1	2	-	-	-
Pomegranate	2	-	3(1)	1	1	-	-	-
Strawberry	3	1	2	1	1	1	-	-
Grapes	2(1)	3	1	-	1	-	-	-
Pineapple	2	1	1	1	1	-	-	-
Sweet lime	2	-	1	-	1	-	-	-
Guava	1	1	1	-	-	2	-	-
Berry	1	1	1	1	1	-	-	-
Lichee	3	2	-	1	-	-	-	1
Custard apple	2(1)	3	1	1	-	-	-	1
Mango	3	-	2(1)	2	1	1	-	-
Watermelon	-	1	-	-	-	1	-	-
Total	**32**	**17**	**22**	**12**	**14**	**6**	**-**	**2**

( ) = reappearance of isolate after 60 days of storage.

Amongst the isolates, 87% isolates were gram positive and 13% were gram negative, 42% showed pigmentation and 44% colonies were opaque. The selected 17 cultures, which gave EPS production more than 0.9 g/L were represented by 83% gram positive bacteria and 17% gram negative bacteria. Biochemical test and sugar utilization pattern revealed that all the isolates showed casein hydrolysis, and oxidative test positive; where as, ammonia production, deaminase test, decarboxylase test and fermentative test were negative. Out of 17 isolates, 6 isolates were able to produce only acid in all 21 sugars. A similarity index of 17 isolates based on biochemical test and sugar utilization pattern is shown in Table 2.

Table 2 Similarity index based on biochemical characteristics and sugar utilization pattern.

	% Similarity																
	Isolates																
Isolates	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
1	1	0.55	0.57	0.64	0.37	0.71	0.6	0.6	0.62	0.37	0.6	0.55	0.62	0.46	0.6	0.46	0.57
		1	0.84	0.86	0.55	0.66	0.80	0.86	0.84	0.73	0.68	0.77	0.77	0.75	0.86	0.66	0.68
zz2																	
3			1	0.93	0.51	0.81	0.95	0.97	0.91	0.68	0.91	0.82	0.88	0.82	0.93	0.68	0.80
4				1	0.53	0.64	0.95	0.93	0.97	0.6	0.88	0.84	0.86	0.82	0.95	0.66	0.75
5					1	0.37	0.55	0.53	0.51	0.6	0.53	0.55	0.57	0.57	0.57	0.46	0.44
6						1	0.57	0.64	0.62	0.46	0.62	0.62	0.57	0.6	0.64	0.57	0.55
7							1	0.93	0.88	0.57	0.86	0.77	0.84	0.82	0.88	0.71	0.75
8								1	0.93	0.64	0.93	0.84	0.86	0.84	0.95	0.73	0.77
9									1	0.57	0.86	0.84	0.84	0.77	0.93	0.71	0.75
10										1	0.57	0.6	0.6	0.66	0.6	0.55	0.48
11											1	0.8	0.84	0.77	0.93	0.68	0.75
12												1	0.75	0.68	0.84	0.68	0.71
13													1	0.75	0.82	0.57	0.68
14														1	0.80	0.62	0.66
15															1	0.68	0.77
16																1	0.66
17																	1

Based on biochemical test and sugar utilization characteristics out of 17 cultures, none of the culture showed 100% similarity. The maximum similarity observed was 97% amongst culture 3 and 8 (C1A and SRA 8), 4 and 9 (SRA 1 and SRA 5), 12 cultures showed more than 80% similarity, out of these 6 cultures showed more than 90% similarity. The similarity index of the isolate (SRA 17) was 37% to 60%; whereas, that of isolate 10 (SRA 15) was 37% to 73%. This data clearly shows the wide diversity of EPS producers isolated from the selected samples.
Based on 16S rRNA partial gene sequence, of selected 17 isolates, *Bacillus subtilis* comprised 25% of the isolates and if genus *Bacillus* is considered, it comes out to be near 60% of the total selected isolates. Remaining 40% are represented by 7 different genus (Table 3).

Table 3 Identification of selected isolates based on 16S rRNA sequencing

Source	Isolate code	Isolate identified	GenBank accession number
Potato peels	SRA 18	*Escherichia coli*	KTG30840
Sweet lime	SRA 6	*Bacillus tequilensis*	KM406457
Chikoo	C1A	*Bacillus subtilis* subspecies *spizizani*	KM406418
Custard apple	SRA1	*Bacillus soneresis*	KM406421
Spoiled orange	SRA 17	*Xanthomonas campestris*	KTG30839
Spoiled custard apple	SRA 9	*Lactobacillus fermentum*	KM406420
Pomegranate	SRA 4	*Bacillus* species	KM406429
Sweet lime	SRA 8	*Bacillus methylotrophicus*	KM406458
Pomegranate (i)	SRA 5	*Bacillus licheniformis*	KM406430
Spoiled guava	SRA 15	*Bacillus subtilis*	KP178604
Chikoo 2	C2A	*Bacillus subtilis* species	KM406419
Spoiled lichee	SRA 3	*Leuconostoc pseudomestenteroids*	KM406428
Mango	SA 100	*Enterobacter cloacae*	
Orange	SRA 2	*Bacillus amyloliquefaciens*	KM406427
Spoiled mango	SRA 7	*Bacillus* species	KM406459
Potato peels 1	SRA 14	*Micrococcus* species	KP178617
Orange	SRD 1	*Panebacillus polymyxa*	KJ830755

Carbohydrates are the major component of the cytoskeleton and an important nutritional requirement for the growth and cell development. Production of EPS was studied using YE broth containing 3% sucrose, glucose, galactose and mannitol as a source of carbon. In this study, it was found that irrespective of selected isolates, production of EPS was more than at least 2 fold higher in sucrose containing media as compared to any other sugar used in the media except isolates SRA 3 and SRA 18 (Fig. 1). EPS dry weight production ranged between 0.9 to 5.4 g/L in sucrose containing medium; whereas, it was 0.06 to 3.79, 0.03 to 0.99, 0.02 to 0.6 g/L in glucose, galactose and mannitol containing medium respectively.

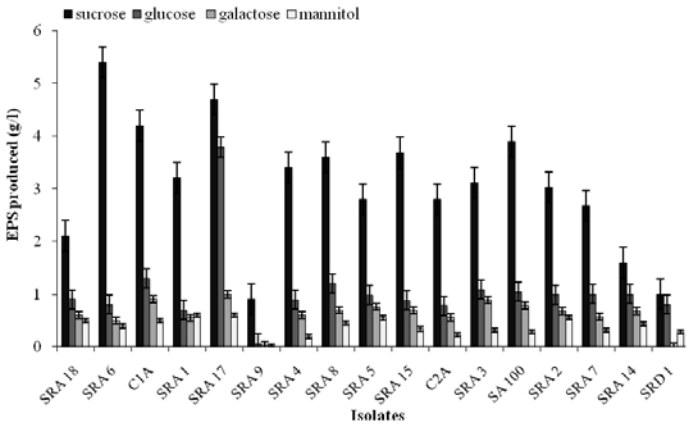

Figure 1 Influence of different sugars on EPS production.

Influence of sucrose concentration on EPS production is shown in Fig. 2. EPS production was 1.06 to 3.8 and 1.2 to 3.5 fold higher in the presence of 5% sucrose as compared to 3% and 7% sucrose in the medium respectively.Variation of EPS production by different isolates in types of sugar used and the concentration of sucrose added in the medium indicates the metabolic and physiological diversity of the isolates and their preference for the sugar as well as concentration of sucrose in the medium.

Comparative study of EPS production between YE broth containing 5% sucrose and EPS medium was also performed. It was observed that constituents of EPS medium gave a higher production of EPS in comparison to Yeast Extract broth except for isolate SRA17. The differences of EPS produced in the EPS medium as compared to SYE were as small as 1.03 and as high as 4.4 fold (Fig.3). The selection of EPS medium as a medium, sucrose as a source of substrate and 5% concentration of sucrose resulted in more than 4.4 fold increase in the EPS production. Amongst selected isolates under the experimental conditions, *Bacillus* species gave higher EPS production as compared to species of *Xanthomonas* and *Leuconostoc* obtained in this study, which are reported to be EPS producer.

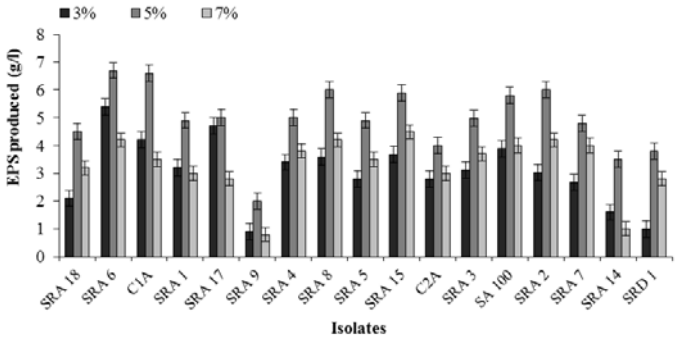

Figure 2 Influence of different sucrose concentration on EPS production.

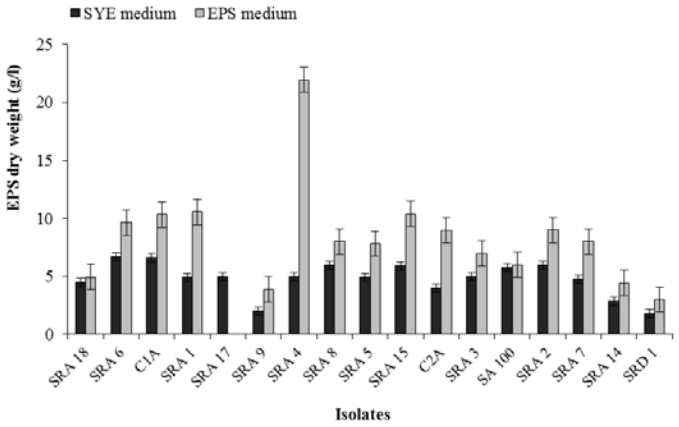

Figure 3 Influence of different medium on EPS production.

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DIESEL BIOTREATMENT COMPETENCE OF INDIGENOUS METHYLPARATHION DEGRADING BACTERIAL STRAIN OF PSEUDOMONAS AERUGINOSA DOU

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ABSTRACT

Pseudomonas aeruginosa methylparathion degrading bacterial strain previously isolated and identified in our lab was subjected to confirm their ability to diesel oil degradation in synthetic wastewater containing diesel oil (10 % v/v). They are cultured in modified mineral salts agar medium with diesel oil as the sole source of carbon. They were able to grow and distinguish in 7 days of incubation. Biosurfactant was produced in the medium and its effect was detected using emulsification activity, growth estimation and gravimetric analysis as an indicator for this process. Factors affecting hydrocarbon utilization by this isolate were pH, temperature, salt concentration and agitation along with glucose, peptone, phosphate and nitrate concentrations. Gravimetric and FTIR analysis showed that the strain was able to degrade complex hydrocarbon chains to simple ones. The optimum conditions of various factors showed that, neutral pH, with temperature of 32^{0}C, and agitation rate of 150 rpm gave the optimum conditions to accelerate diesel oil degradations, while additional nutrients of glucose (1 mg/l), peptone (5 mg/l), sodium nitrate (2 mg/l), phosphate (7 mg/l) and sodium chloride (3 mg/l) enhanced optimum effect on the bioprocess. The bacterial strain of indigenous pure monoculture having the degradative potential of xenobiotic compounds of various pollutants includes diesel oil as well as methylparathion pesticide practically. The main conclusion is that *Pseudomonas aeruginosa* dou-1 strain can be able to degrade complex hydrocarbons and make it an ideal applicant in bioremediation.

Keywords: Methylparathion tolerant strain, *Pseudomonas aeruginosa* dou-1, diesel oil, bioremoval, FTIR, wastewater

INTRODUCTION

Environmental pollution with petroleum and petroleum products (complex mixture of hydrocarbons) has been recognized as one of the most serious current problems, hydrocarbons may reach the water table before becoming immobilized in the soil. They spread horizontally on the ground water surface and continue to partition into ground water, soil pore space, air and to the surface of soil particles. Oil contamination of soil and water from industrial sources and other activities are producing ecological disasters and addressing public concerns (**Che, 2002**). India's oil and gas sectors meet around 42% of the country's primary energy demand and contribute over 15% to the gross domestic product. Oil spill accidents are very common in the areas near the seashore because of shipping of oil tankers and bursting of oil supply pipelines. Recently in India, mumbai-urban oil spill through the pipeline bursting witnessed about 600 metric tons spillage of crude oil which caused a widespread mortality of sea animals. The contamination of soil and groundwater by hazardous chemicals has become a major concern due to the associated risks to human health and the environment (**Ghosh and Tick, 2013**).

Petroleum-based products are the major source of energy for industry and daily life. Petroleum products such as engine oil, petrol, diesel and kerosene are used daily in various forms in mechanic workshops. Petroleum hydrocarbons composed of complicated mixtures of non-aqueous and hydrophobic components such as n-alkane, aromatic, resins and asphaltenes. Oil contamination with petroleum hydrocarbons has caused critical environmental and health defects and increasing attention has been paid for developing and implementing innovative technology for cleaning up this contamination (**Yeung, 1997**). Diesel, a derivative of Petroleum is one of the major pollutants of water and soil. In areas where oil refineries are present, air, water and soil resources have become contaminated with oil and its by-products namely diesel. Diesel oil, which is one of the major products of crude oil, constitutes a major source of pollution in our environment. Transportation through the railways and roadways causes organic and inorganic contamination. Broken and corroded oil pipelines significantly contribute to oil pollution on a large and rapidly increasing scale. Due to the adverse impact of these chemicals on human health and environment, they are classified as priority environmental pollutants by the US Environmental Protection Agency (1996). The numbers of spills that have dumped millions of gallons of crude oil into the environment have been steadily increasing over the past decade. This can lead to disastrous consequences. Therefore the pollutants need to be removed or destroyed from the environment.

An array of procedures has been developed including physical, chemical and biological techniques. Among these procedures, bioremediation is currently used alone or associated with physicochemical procedures. Biological methods of rehabilitation of polluted sites represent an interesting alternative. These techniques are based on the microorganism's capacities to degrade petroleum compounds (**Harayama, 1999**). Bioremediation has become an alternative way to remedy oil polluted sites, where the addition of specific microorganism (bacteria) or enhancement of microorganism already present can improve biodegradation efficiency (**Hagwell *et al*.**, 1992). These microorganisms can degrade a wide range of target constituents present in oil sludge (**Barathi and Vasudevan, 2001; Mishra *et al*., 2001**).

The study of petroleum degrading bacteria therefore becomes very important in deal with problems of oil and petroleum based pollutions. In this study, *Pseudomonas aeruginosa* mehtylparathion degrading bacterial strain previously identified and stored in our lab was subjected to diesel oil degradation in synthetic wastewater containing diesel oil.

MATERIAL AND METHODS

The mehtylparathion degrading *Pseudomonas aeruginosa* bacterial strain previously identified and stored in the lab was used for diesel oil degradation. Diesel oil used in this study was refined diesel oil commercially available, which was purchased from local petrol station.

Morphological and Biochemical Characterization

Gram staining revealed that the morphological characters of the isolated bacterial strains. Spore staining shows weather the organism is spore producing or not. Motility test can determine the organism which is motile or non-motile. Catalase test was performed to check the ability of the isolated strains to degrade hydrogen peroxide and Oxidase test for the detection of presence of cytochrome oxidase enzyme. IMViC test is performed for the identification of the organisms. Carbohydrate utilization tests revealed that the ability of the isolated strains in fermentation of sugars like glucose, lactose, sucrose and mannitol. The genus and species of *Pseduomonas aeruginosa* was identified and confirmed by *Pseudomonas* agar medium and King's A medium. The identification was done by on the basis of morphological and biochemical characteristics as per Bergeys Manual of Systemic Bacteriology (Holt et al., 1994).

Screening the capability of diesel oil utilization by potential methylparathion degrading bacterial strain

The screening of the diesel oil degrading bacteria was done by inoculating 1 ml of pure culture (*Pseudomonas aeruginosa*) in diesel oil containing nutrient broth (NB) and incubated for 21 days at 35ºC on rotary shaker at 150 rpm. After growth in NB, serial dilutions (up to 10^{-5}) were made and 100µl of the liquid was surface spread on nutrient agar plates containing diesel oil (1% v/v) and plates were incubated at 35ºC for 72h. A colony was picked and streaked on nutrient agar plates with increased concentration of diesel oil (up to 10%). Out of these the best grown isolates were selected and were maintained in modified mineral salts (MMS) agar medium ($K_2HPO_4$7.0 mg/l; $NaNO_3$ 2.0 mg/l; NaCl 3.0 mg/l; Peptone5.0mg/l; glucose 1.0 mg/l; agar 22.0 mg/l; Distilled water 1000 ml; pH-7.0 ±0.2) and stored at 4 ^{0}C for further work.

Halo plate diffusion assay

Growth on diesel was determined by halo plate diffusion assay by Bushnell Hass (BH) agar medium (**Geetha *et al.*, 2013 and Saadoun, 2002**). Colonies of the different bacterial isolates were transferred into 50 ml BH Medium (**Leadbetter, Foster, 1958**), supplemented with 10 % (v/v) diesel oil solutions, filter sterilized through 0.45µ membranes and incubated at 35°C in a water bath shaker at 150 rpm for 9 days. Bacterial growth was determined at intervals by measuring the optical density (OD_{600} nm). The test to detect the biodegradation of diesel was studied by a monooxygenase biodegradation pathway (**Jacob *et al.*,1983**). The test was performed at 28ºC in a glass test tubes containing; 20 µl of 0.05 mol/l 2,6- dichlorophenolindophenol (DCPIP) (Himedia, India); 30 µl of 0.05 mol/l 5 methyl-phenazinium methylsulphate (5-MPMS); 25 µl 0f 0.1% (v/v) diesel; 5 µl of 0.15 M NAD solution and 25 µl of pre-washed bacterial cells. Change in color was visually compared with different controls: no diesel (substrate); no NAD+; no cells and heat-killed cells. The reaction was monitored up to 12 hours with intermittent observations (**Geetha *et al.*, 2013**).

Optimization of growth conditions

The initial enrichment cultures were established in synthetic wastewater containing mineral salts medium amended with the diesel oil as the sole source of carbon and energy. The optimum temperature, agitation, pH and salinity concentration, glucose, peptone, nitrogen, nitrate, phosphate and diesel concentrations for the growth of the bacterial strains were determined. In order to optimize the growth conditions, potential bacterial strains (1% v/v) was grown in a conical flask containing 100 ml MMSM and 1% (v/v) diesel at 35ºC for 24 hr and it provides as the inoculum. The MMSM containing flaks were inoculated with 1ml of inoculum with 0.1 OD culture and incubated the flasks at 35ºC for 9 days. After every 24 hr the treated culture medium was analysed for the diesel degradation up to 9 days. The growth in terms of optical density (OD_{600nm}) value was determined by turbidity measurement using UV-Vis spectrophotometer (Shimadzu - UV- 3600, Japan).

Effect of different factors on diesel oil utilization

Different factors were tested for optimization of diesel utilization. These were pH, NaCl concentration, temperature, time and agitation (Satpute *et al.*, 2008).

Effect of diesel oil concentration

The concentration of diesel oil as hydrocarbon source (up to 10% v/v) was studied to determine the effect of diesel oil on the growth of bacteria.

Effect of Temperatures on growth.

Temperature is one of the most important physical factors affecting bacterial growth. The potential bacterial strain inoculated MMS medium containing flasks along with control were incubated for 9 days at different temperatures (28ºC, 30ºC, 35 ºC and 40ºC) in a rotary shaker at 150 rpm. The presence or absence of growth of the cultures was observed after every 24 hr of incubation. The degree of growth was observed at wavelength of 600 nm using spectrophotometer.

Effect of agitation on growth

The effect of agitation on the growth of bacteria in MMSM was varied at different rpm (100, 120, 150 and 170 rpm) using incubator shaker and the optimum growth was monitored.

Effect of pH on growth

To study the effect of pH on the growth of bacteria, the pH of the MMSM was varied (pH 5, 6, 7, 8, 9) using 1N HCl and 1N NaOH and the growth was continuously monitored. The pH was adjusted and monitored using pH meter (Fisher Accruement pH meter).

Effect of salt concentrations on growth.

The effect of salt concentration on the growth of the bacteria was studied by growing the culture in MMSM with different salt concentration (4%, 8%, 12%, 16% and 20% of NaCl). One ml of the potential culture strain was added into the MMSM with different salt concentration and incubated for 24-48 hours at 35ºC in a rotary shaker at 150 rpm.

Effect of carbon source and glucose concentration on growth

The effect of carbon source was investigated by growing the culture in MMSM supplemented with different carbon sources includes glucose, sucrose and fructose. Glucose was selected as the source which can best enhance the growth of bacteria. The concentration of the glucose was varied (0.5 %, 1 %, 2%, 3% w/v) to probe the influence of concentration of carbon on the growth of bacteria.

Effect of nitrogen source and its concentration

The effect of nitrogen source was investigated by growing the culture in MMSM supplemented with different nitrogen sources; urea, ammonium nitrate, sodium nitrate. Sodium nitrate was selected as the nitrogen source which can best enhance the growth of the bacteria. The concentration of the sodium nitrate was varied (0.5%, 1%, 2%, 3% w/v) to probe the influence of concentration of nitrogen on the growth of bacteria. The effect of peptone concentration was also studied with various concentration of peptone (0.5, 1-6 mg/l) to investigate the growth of bacteria.

Screening of oil degrading Biosurfactant producing bacterial strains

The isolates of the bacteria was screened for production of oil degrading biosurfactant. The screening was carried out by the following methods. Biosurfactant production and diesel degradation capabilities of the isolates were determined by using drop collapse method, oil spread method and measurement of emulsification index (**E_{24}**). The determination of diesel oil degradation ability of the selected potential bacterial strain was done by gravimetric analysis and FTIR analysis.

Drop collapse method

Biosurfactant production was screened using the qualitative drop collapse test. 2µl of diesel was added to 96 well micro-titre plates. The plates were equilibrated for 1 hour at 30ºC and 5µl of the respective culture supernatant obtained was added to the surface of the oil in the well. The shape of the drop on the oil surface was observed for 1 minute. If the culture supernatant makes the drop collapse, it indicated positive result for biosurfactant presence and if the drop remained as such it indicated negative result (**Bodour et al., 2003**).

Oil spread method

The petridish base was filled with 50 ml of distilled water. On the water surface, 20µl of diesel and 10µl of culture were added respectively. The culture was introduced at different spots on the diesel which is coated on the water surface. The occurrence of a clear zone was an indicator of positive result (**Morikawa *et al.*, 2000**).

Emulsification Index (E_{24})

The emulsifying capacity was evaluated by an emulsification index. The E_{24} of the culture samples was determined by adding 2 ml of diesel and 2 ml of the inoculum in a test tube and it was shaken for 2 minutes. Then, water and diesel were added and shaken for 2 minutes to obtain maximum emulsification and allowed to stand for 24 hours. This was taken as control (**Desai and Banat, 1997**).

The percentage of the E_{24} index is calculated by the following formula:

eqn.[1]

$$E_{24} = \frac{\text{Height of the emulsified layer (cm.)}}{\text{Total height of the column (cm.)}} \; x\; 100$$

Analytical Characterization

Batch degradation of diesel oil by potential bacterial strains in SWW

Biodegradation of diesel oil using *Pseudomonas aeruginosa* dou-1 was evaluated in synthetic wastewater. The potential bacterial strains at the log phase overnight cultures with 1 O.D (at 600 nm) were transferred to 250 ml conical flasks containing 100 ml of sterile defined modified mineral salts medium with 10% diesel oil in triplicates. All flasks were incubated at 32°C in a rotary shaker at 150 rpm for 10 days. The uninoculated medium containing 10% diesel oil and MMSM broth serves as control. At 24 hr intervals, sets of flasks were used for the extraction of residual oil. Whole sample contained in the individual flasks were extracted at initial 0 time, and every 24 hrs up to 10 days for the purpose of measuring residual concentration (**Bishnoi *et al*., 2009**). The total hydrocarbons in the treatments were determined gravimetrically.

Gravimetric analysis

The oil degradation was quantified by measuring the oil recovery after 16th days of incubation using the gravimetric analysis (**Marquez-Rocha *et al.*, 2001**). The amount of oil in wastewater was estimated using the Gravimetric method. About 4ml of treated culture sample was taken from flask. Petroleum ether and acetone were taken in the ratio 1:1 and was mixed with the sample in a separating funnel. The mixture was shaken for about 45 minutes and then was left undisturbed for about 10 minutes. The upper solvent along with oil was separated from the lower extract. The solvent with the oil layer was then kept in the hot air oven at about 50º C until the solvent gets evaporated. After the complete evaporation, the oil residue obtained was weighed and taken as the gravimetric value for further calculation. Analysis of sample before and after treatment was done using this Gravimetric method.
The percentage of diesel oil degraded was determined from the following formula:

eqn.[2]

$$\text{PDOD} = \frac{\text{Weight of diesel oil degraded}}{\text{Weight of diesel oil present originally}} \; x\; 100$$

Where,
PDOD = Percenetage of diesel oil degradated,
the weight of diesel oil degraded = (original weight of diesel oil) – (weight of residual diesel oil obtained after evaporating the extractant).
Weight of residual oil = (Weight of beaker containing extracted oil) – (Weight of empty beaker).

FT-IR Analysis

After 7 days of growth, cell biomass was removed by centrifugation of broth culture at 10, 000 rpm for 15 minutes. Then the supernatant was precipitated overnight with ice cooled ethanol. The precipitated medium was centrifuged and the pellet was collected, dialyzed against distilled water for 24 hours and then lyophilized. A lyophilized sample was ground with potassium bromide (KBr) powder and pressed with 7,500 kg for 30s to obtain a translucent pellet. The infrared spectra were recorded on an FT-IR system (Perkin Elmer 783, Germany) within the range of 400-4,000 cm^{-1} wave number. A KBr pellet was used as background.

RESULTS AND DISCUSSION

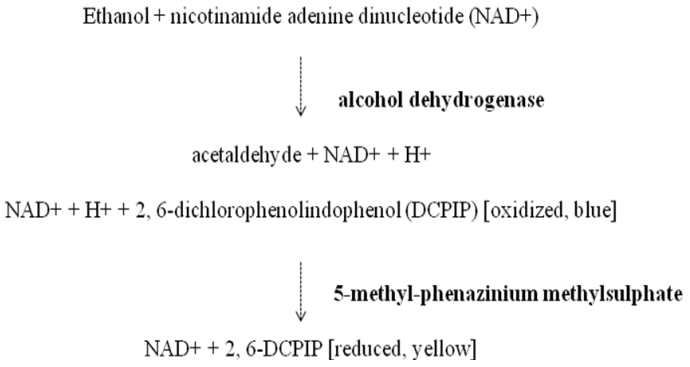
Morphology and Biochemical Characterization

The selective potential bacterial strain was analyzed taxonomically. The colony morphology of the strain was abundant, thin, white growth with medium turning green, earthy odour and rod shape. The strain was found to be Gram negative and rod shaped bacterium (Figure. 1). Based on the biochemical characteristics, the selected potential bacterial strain was identified as *Pseudomonas aeruginosa* dou-1(Table. 1).
Screening the competence of diesel oil utilization by potential methylparathion degrading bacterial strain (*Pseudomonas aeruginosa dou-1*).
The bacterial strain used for the biotreatment process was *Pseudomonas aeruginosa* dou-1 (Table.1 and Figure 1), which is a methylparathion degrading bacterial strain previously identified according to Bergy's manumal of determinative bacteriology (**Holt *et al.*, 1994; Cappuccino and Sherman, 2010**) stored in the lab was subjected to diesel oil degradation in synthetic wastewater containing diesel oil at 10% (MMSM). The bacterial strain which was found to persist and grow under high diesel oil concentration was screened from this study and named as *Pseudomonas aeruginosa* dou-1. In Figure.1, there was not much difference between the colony forming units found in nutrient agar (NA-36 $x10^{-2}$ CFU/ml) was) medium when compared to modified mineral salts agar medium (MMSM- 34 $x10^{-2}$ CFU/ml).

Table 1 Identification of potential bacterial strain of biosurfactant activity

Biochemical Tests	Characters
Colony morphology on Nutrient Agar medium	Abundant, thin, white growth with medium turning green, earthy odour
Gram stain	Negative, Rod shaped
Spore	Negative
Motility	Positive
Catalase	Positive
Oxidase	Positive
Indole	Positive
Methyl Red	Positive
Voges Proskaur	Positive
Citrate	Positive
Nitrate	Positive
Glucose	Positive
Lactose	Negative
Sucrose	Negative
Mannitol	Negative
Rhamnose	Negative
H_2S production	Negative
Gelatine liquefaction	Positive
Casein hydrolysis	Negative
Lipid hydrolysis	Positive
Starch hydrolysis	Negative
Growth: at (28 - 40°C)	Positive
at (pH 5– 8)	Positive
at (100-170 rpm)	Positive
Growth in Selective medium Cetrimide agar medium	Positive
Pigment production: Pseudomonas agar medium and King's A medium.	Positive
Identified Bacterial strain	***Pseudomonas aeruginosa* dou-1**

Hole-plate diffusion test using diesel as a carbon source showed bacterial growth around the holes containing the diesel. Growth of hydrocarbon degrading bacteria using different concentration of hydrocarbons as carbon source was analysed. The bacterial growth was observed in different concentration of hydrocarbons upto 10%. The potent hydrocarbon degrading isolate (based on hole-plate diffusion method) were selected for the diesel degradation assay, by a monooxygenase biodegradation pathway. The test is based on the following reactions (**Geetha *et al*., 2013**).



But while considering the economic feasibility and applicability in wastewater treatment, the MMS medium was selected for further biotreatment study. The isolates grew maximally on the diesel substrate when supplied as the sole source of carbon and energy. In a similar research by **Rahman et al. (2002),** the total viable count method was used to confirm the potential of different kind of bacteria utilizing hydrocarbon. This technique was used in several studies to show the ability of bacteria utilizing hydrocarons (**Emtiazi and Shakarami, 2004**).

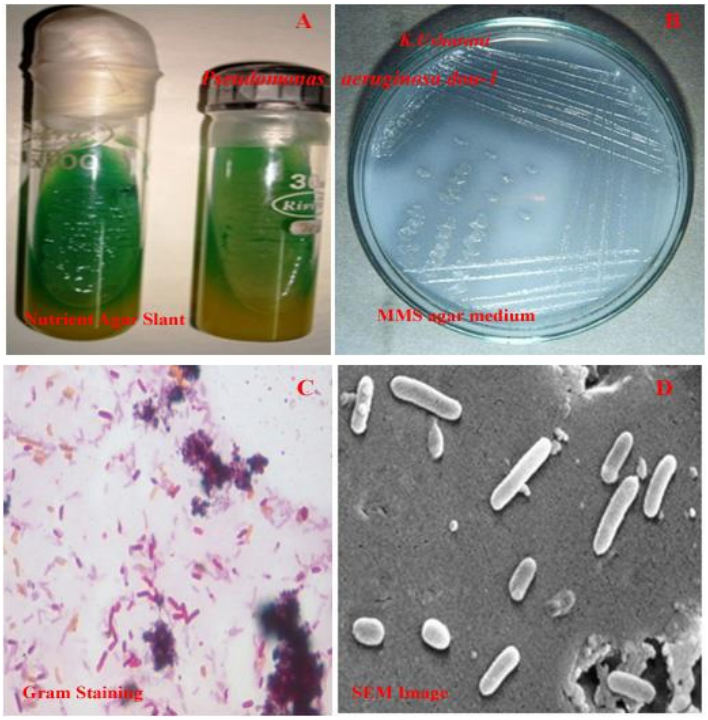


Figure 1 Identified potential bacterial strain of *Pseudomonas aeruginosa dou-1* (A) on NA slant (B) on MMS agar medium (C) Gram Staining (D) SEM Image

Optimization of growth conditions

Effect of diesel oil with respect to time on the growth of selected bacterial strain (*Pseudomonas aeruginosa* dou-1)

The effect of different concentration of diesel oil (1, 2, 4, 6, 8 and 10 %) on the growth of potential bacterial strain was studied. The bacterial strain *Pseudomonas aeruginosa* dou-1 showed an increase in cellular density with respect to growth tolerance occurring at maximum 10 % (v/v) of diesel concentration (Figure.2A). Cell density considerably reduced at diesel concentrations higher than this. The selected bacterial strain *Pseudomonas aeruginosa* dou-1 was able to tolerate high diesel concentration; this suggests that the strain is a better applicant for diesel biodegradation. Biodegradation studies on diesel are carried out using diesel concentrations ranging from 0.5 to 1.5% (**Mukherji *et al*., 2004; Hong *et al*., 2005; Lee *et al*., 2006; Ueno *et al*., 2007**). Degradation at a much higher concentration (6% v/v diesel) has been reported but degradation requires glucose (0.2% w/v) and yeast extract (0.1% w/v) (**Kwapisz *et al*., 2008**). Diesel is essential as a carbon source but it can be toxic to microorganisms due to the solvent effects of diesel that could obliterate cell membrane (**Shukor *et al*., 2009**).

Effect of agitation with respect to time on the growth of bacteria

The effect of different rate of agitation (rpm) on diesel degradation from the oil contaminated wastewater was studied. The bacterial strain *Pseudomonas aeruginosa* dou-1 showed optimal growth at 150 rpm (Figure. 2B).

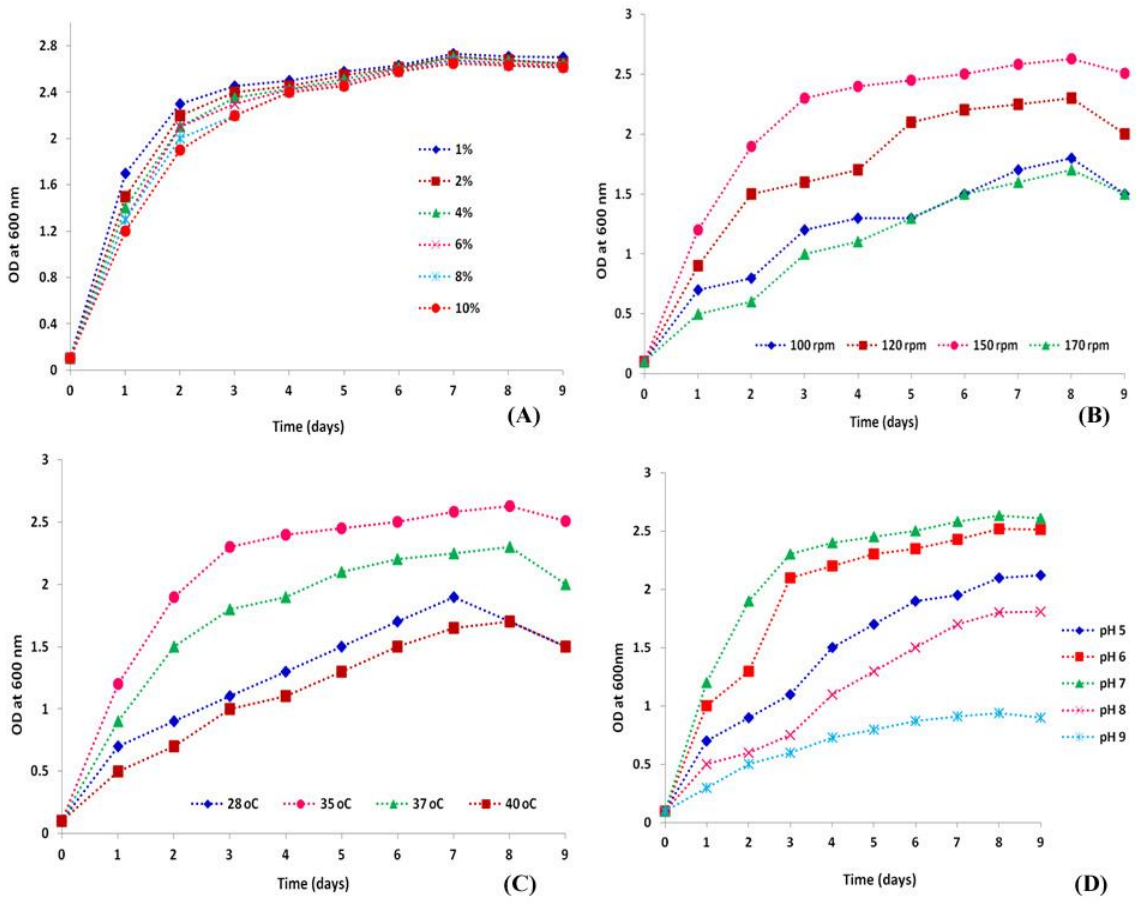


Figure 2 Effect of initial diesel oil concentration, agitation, temperature and pH on the growth of *Pseudomonas aeruginosa* dou-1

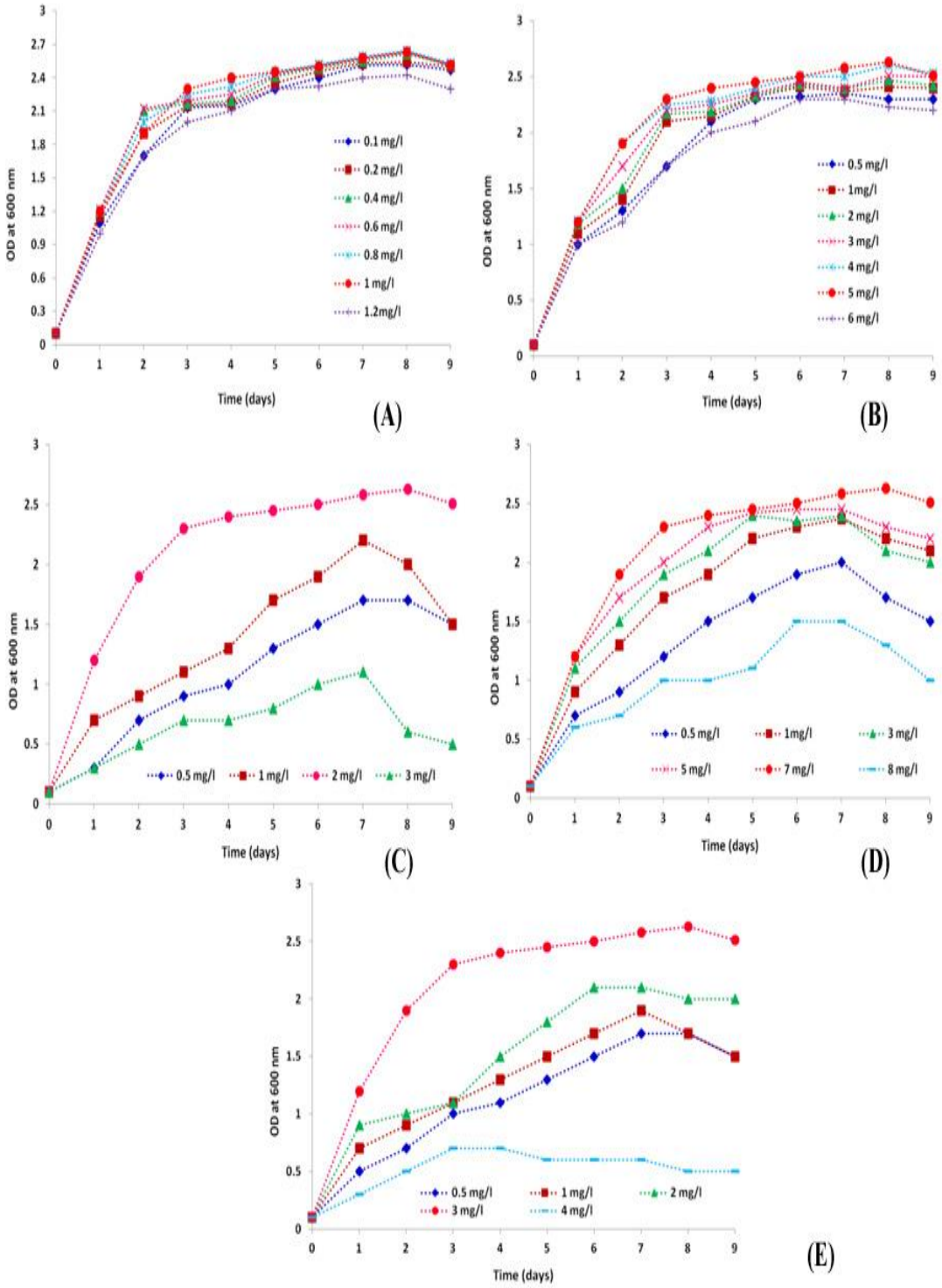


Figure 3 Effect of glucose, peptone, nitrate, phosphate, sodium chloride concentration, on the growth of *Pseudomonas aeruginosa* dou-1

Effect of temperature with respect to time on the growth of bacteria

Among factors limiting microbial activity, temperature is one of the most significant (**Atlas and Bartha, 1993**). The effect of different temperature on diesel degradation in wastewater was studied. The bacterial strain *Pseudomonas aeruginosa* dou-1 showed optimal growth at 35^{o}C (Figure. 2C). The influence of temperature on the biodegradation of diesel oil was favourable at optimum temperature of 35^{o}C.

Effect of pH with respect time on the growth of bacteria

The optimization of growth conditions showed that pH 7 was the best for the growth of *Pseudomonas aeruginosa dou-1* (Figure. 2D) and followed by pH6, pH5, pH8. Optimal growth of the strain *Pseudomonas aeruginosa* dou-1 occur at pH 7.0 in MMS medium with significantly higher growth was attained. Minimal growth was observed at pH 9.0 and at pH 5.0 (Figure. 2D).

The isolate showed an optimum growth requirement indicating that pH control is very important to get optimum results during bioremediation. Therefore, the optimization of environmental conditions is important for the enhancement of bacterial growth and for designing effective bioremediation strategy (**Davey, 1994**). The requirement of neutral or near neutrality for optimal growth of bacteria on diesel is also exhibited by many other diesel-degrading bacteria (**Marquez-Rocha *et al*., 2005; Rajasekar *et al*., 2007; Kwapisz *et al.*, 2008**).

Effect of glucose concentration on the growth of bacteria

To study the effect of carbon and nitrogen sources on degradation, MMSM supplemented with 10 % of diesel oil along with carbon source such as glucose and nitrogen source such peptone. The effect of different glucose concentration

on the growth of bacterial strain for diesel degradation in wastewater showed glucose concentration of 1mg/l gave up the optimum growth of *Pseudomonas aeruginosa* dou-1 (Figure.3A). Glucose served as the most favourable carbon source in comparison with sucrose and fructose. The carbon source is mainly used as a cellular constituent, and for synthesis of new cells and production of polysaccharide as an energy source.

Effect of peptone concentration on the growth of bacteria

The effect of different peptone concentration on the growth of bacterial strain for diesel degradation in wastewater confirmed that the optimal concentration that enhances the maximal growth was found to be 5 mg/l (Figure. 3B). The peptone rich of nitrogen source influence the growth and metabolism of the selected strain of *Pseudomonas aeruginosa* dou-1.

Effect of nitrate concentration on the growth of bacteria

The nitrogen source affects the growth and metabolic activities of the organism. Various nitrogen sources such as urea, ammonium nitrate, sodium nitrate were used at an initial concentration of 1% (w/v) in MMS media supplemented with up to 10 % diesel to study their effects on bacterial growth. Sodium nitrate was observed to be the most suitable nitrogen source for the growth of the tested bacterial strain. The effect of different concentration of sodium nitrate on the growth of bacterial strain for diesel degradation in oil contaminated wastewater showed that the optimal concentration that supports maximal growth was at 2 mg/l (Figure. 3C). Sodium nitrate was selected as the nitrogen source which can best enhance the growth of the bacteria, was chosen as the principal nitrogen source due to its widespread usage as a cheap source of nitrogen for bioremediation (**Sugumar *et al*., 2014**).

Effect of phosphate concentration on the growth of bacteria

The effect of different concentration of phosphate on diesel degradation from the oil contaminated wastewater confirmed that the optimal concentration that supports maximal growth was at 7 mg/l (Figure. 3D).

Effect of salt concentration on the growth of bacteria

The effect of different NaCl concentration on the growth of bacterial strain for diesel degradation in wastewater showed that increasing NaCl concentration in wastewater had decreasing effect on diesel degradation. The amount of oil degraded by *Pseudomonas aeruginosa* strain increased initially to a maximum level at 3 m/l NaCl, but thereafter decreased with increasing salt concentration for the substrates (Figure. 3E).

Growth and screening of oil degrading biosurfactant producing bacterial strain *Pseudomonas aeruginosa* dou-1 in MMSA medium at optimum condition

From the Figure.4A, the growth in terms of optical density (2.68 OD) was found to be optimum on 7th day. Thus bacterium growth reached the stationary phase and moved into the death phase and this is probably due to the chemistry of the diesel degradation. The utilization of the diesel oil as sole carbon and energy source by the isolate resulted in the growth with a resultant production of acid. This is probably as a result of chemical change of the diesel oil, hydrocarbons and production of by products and ability of isolated *Pseudomonas aeruginosa* dou-1 to use diesel oil and generates organic acids. The biodegradation effectiveness of diesel oil in terms of growth by bacterial strain *Pseudomonas aeruginosa* dou-1 increased in efficiency from the 1st day to 7th day of incubation, increasing from 1 to 10 % of DO. Moreover, the growth studies indicated that diesel degradation ability was correlated with cellular growth of the organism. Almost complete removal of diesel oil (83%) as seen after 7days of incubation. The initial pH of the medium used in this experiment was neutral; a decreasing trend of pH was detected in the experimental flasks within the incubation period, as growth increases in the presence of the diesel oil was observed (Figure.4B). A change in pH is largely due to the production and accumulation of bacterial waste products. Maintenance of pH is important since pH strongly affects bacterial growth (**Sugumar *et al*., 2014**).

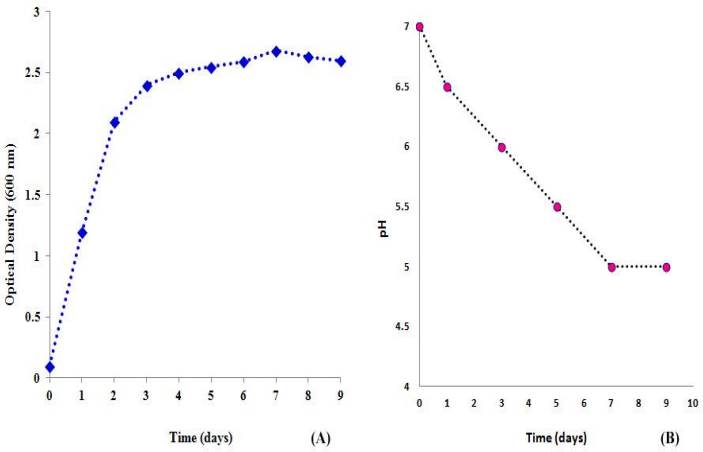


Figure 4 (A) Growth of diesel oil utilizing bacterial strain *Pseudomonas aeruginosa*dou-1 and (B) pH change in culture medium (MMS) at optimized conditions after biotreatment

Drop collapse method

The bacterial strain selected out of enrichment technique in MMS agar medium was used for screening of biosurfactant production purpose. A significant biosurfactant activity was reported when *Pseudomonas aeruginosa* dou-1 strain was used for analysis (Table.2). **Youssef *et al.* (2004)** stated that drop-collapse test expresses the surface activity and indicates production of biosurfactants.

Oil spread method

The study revealed a high biosurfactant activity of the bacterial strain (*Pseudomonas aeruginosa* dou-1) by oil spread method (Table.2). The increase in biosurfactant activity was also reported by other researchers (**Ranjana Julias and Jeya Rathi, 2013**).

Emulsification index(E_{24})

The release of biosurfactants is a strategy used by microorganisms to affect the uptake of hydrocarbon compounds (**Obayori *et al*., 2009**). Consequently, measurement of emulsification activity (E24) experiment was conducted for all of the bacteria which were successful at drop–collapse and oil spread (displacement) tests. The emulsifying capacity of the selected bacterial strain was evaluated by an emulsification index (E_{24}). The emulsification activity test revealed that the emulsification index (E_{24}) was observed to be maximum 72.72% for the diesel oil . The bacteria with emulsification indices higher than 50 % have been defined as potential biosurfactant producers (**Rodriguez-Rodriguez *et al*., 2012**). This method was reported previously by many researchers in their investigations (**Sugumar *et al.*, 2004 and Grazyna *et al*., 2006**).

Table 2 Evaluation of biosurfactant activity by drop collapse and oil spread method

Bacterial strain	Drop collapse method	Oil spread method
Pseudomonas aeruginosa dou-1	positive	positive

Biodegradation efficiency

Diesel oil degradation ability of the isolates was investigated by using both gravimetric and FTIR analysis.

Gravimetric analysis of oil degradation

Gravimetric analysis was used to determine the diesel degradation ability of the potential bacteriium and it was found to be 83% degradation by *Pseudomonas aeruginosa* strain (dou-1). Even though the gravimetric analysis is not as sensitive as GC analysis, it is comparatively helpful method for the preliminary determination. Therefore, the isolated bacteria found out to be successful in preceding experiments were subjected to gravimetric analysis of diesel oil degradation. The residual diesel oil amounts in samples were calculated by using the equation (2). **Olsen *et al.* (1999)** reported 75% for the n-alkane fraction of total extractable petroleum hydrocarbons in diesel oil after 35 days in batch experiments.

FTIR Analysis

The functional group or the chemical species of the biosurfactant was examined using FTIR analysis (Figure 5). The wave number at 2955, 2924 and 2852 cm^{-1} indicate the C-H stretching vibrations of hydrocarbon chain position.

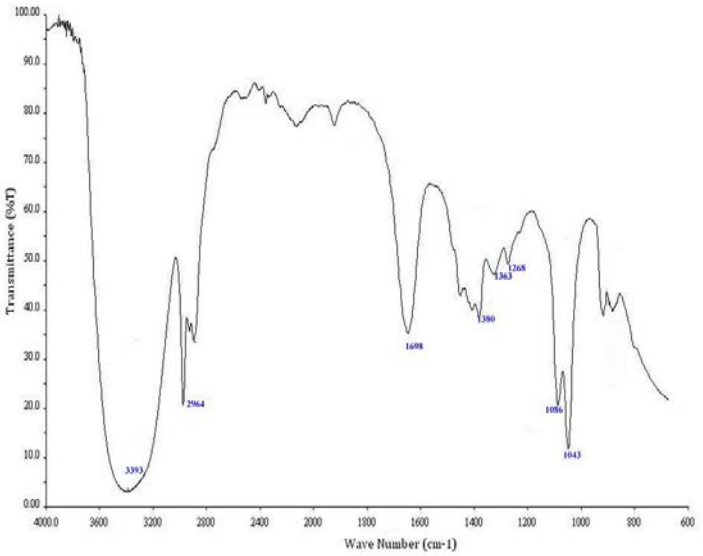


Figure 5 FTIR analysis of biosurfactant produced by the potential bacterial *Pseudomonas aeruginosa* dou-1

The bands at 2964 cm^{-1} resulting from the C – H stretching mode indicate the fatty acid part of lipopeptide. 1268 cm^{-1} exhibits the C-N stretching in peptide bond (amide III band frequency). Hydrogen bending on an aromatic ring could be observed at 800 cm^{-1}. The wave number at 1363 cm^{-1} indicates the chemical structure identical to those of rhamnolipids which are composed of rhamnose rings and long hydrocarbon chains. 1268 cm^{-1} exhibits the C-N stretching in peptide bond (amide III band frequency). The peaks in the range of 1080-1043 cm^{-1} corresponded to C-O-C stretching in the rhamnose. Similarly **Pornsunthorntawee *et al.* (2008)** and **Bondarenko *et al.* (2010)** reported similar results. The use of pure cultures in this study, in addition provides practical advantages by removing the ambiguity associated with constantly variable microbial populations (**Ghazali *et al.*, 2004**). There is a possibility to increase the level of degradation by increasing the number of degradation days. The diesel oil was degraded by *P. aeruginosa* to utilize oil as the sole source of carbon and energy (**Mandri and Lin, 2007**). Hydrocarbon does not have any lethal effect on the growth of the bacteria. This may be due to the ability of the organisms to produce exopolysaccharises, which may protect them from the lethal effects of the hydrocarbons.

CONCLUSION

Soils contaminated with hydrocarbon are good sources for the isolation of diesel oil (DO) degrading bacteria which can then be used for the elimination of such compounds from the environment. The selected bacterial strain in this study from pesticide contaminated soil was recommended that the isolates may be well adapted for use because of prior exposed and acclimatized to the xenobiotic contaminants, which can be used efficiently in bioremediation of the diesel oil contaminated sites. Screening of the isolates (*Pseudomonas aeruginosa* dou-1) at different pH, temperature and agitation indicates the optimum pH of 7, temperature of 35^{0}C and agitation of (150 rpm) in modified mineral salts agar medium at 10% diesel oil (v/v) with optimum concentration of additional nutrients of glucose (1 mg/l), peptone (5 mg/l), nitrate (2 mg/l), phosphate (7 mg/l) and sodium chloride (3 mg/l). The bioremoval and its biodegradation efficiency of *Pseudomonas aeruginosa* was 83% after seven days of incubation. From the study, it was concluded that the bacterial strain having the degradative potential of various pollutants include diesel oil as well as methylparathion. The bacterial strain has the ability to degrade the xenobiotic compounds practically in indigenous pure monoculture.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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BIOPROTECTIVE POTENTIAL OF BACTERIOCINS FROM SOME *Lactobacillus* species ISOLATED FROM FOODS

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ABSTRACT

Lactobacillus species isolated from *ogi*, *kunnu*, yoghurt and palm-wine were found to produce bacteriocins. The bacteriocins had broad spectra of antimicrobial activities against both Gram-positive and negative bacteria. The effects of the bacteriocins on *Escherichia coli* infections in rats were evaluated. *Sprague-Dawley* rats were infected with *E. coli* and treated with 1280 AU/ml of the bacteriocins from *L. plantarum* MO21, *L. plantarum* MP12, *L. casei* MK21, *L. casei* MO11, *L. brevis* MK11 and *L. buchneri* MY21. *Escherichia coli* infection caused upregulation of aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, total protein, globulin, cholesterol, bilirubin and glucose levels in sera of the infected rats which were down-regulated in the bacteriocin treated rats. Gastric and GIT damage caused by *E. coli* infection were reduced in the bacteriocin-treated groups. Therefore, it is concluded that these bacteriocins may have useful biomedical applications.

Keywords: *Lactobacillus* species; bacteriocin; gastric tissue; total protein and globulin

INTRODUCTION

The lactic acid bacteria (LAB) are rod- shaped bacilli or cocci characterized by an increased tolerance to a lower pH range. This aspect partially enables LAB to outcompete other bacteria in a natural fermentation, as they can withstand the increased acidity from organic acid production e.g. lactic acid. Lactic acid bacteria are generally recognized as safe (GRAS) and play an important role in food and feed fermentation and preservation either as the natural micro-flora or as starter culture added under controlled conditions. The preservative effect exerted by lactic acid bacteria is mainly due to the production of organic acids such as lactic acid which result in lowered pH (**Daeschel, 1989**). Lactic acid bacteria also produce antimicrobial compounds including hydrogen peroxide, $C0_2$, diacetyl and bacteriocin (**Cintas *et al*., 2001**).
In probiotic and biotherapeutics, some strains of *Lactobacillus* and other lactic acid bacteria possess potential therapeutic properties including anti-inflammatory and anti-Cancer activities as well as other features of interest (**Arimah and Ogunlowo, 2014**). *Lactobacilli* are considered to have probiotic uses. Many people take *L. acidophilus* to help maintain the pH level of the intestine through the production of lactic acid, which allows for the proliferation of sensitive yet beneficial microbes that are important parts of the fecal floral. *L. acidophilus* is also used as a feed additive for livestock because it supposedly helps the digestibility of feeds through the production of certain enzymes. University of Nebraska research has shown, in the largest feeding study ever conducted that calves fed with feed supplemented with *L. acidophilus* had up to 80% less *E. coli* in their manure. This is the most promising method in inhibiting *E. coli* in livestock to date (**Altermann *et al*., 2005**).
Gastrointestinal disorder is caused by various factors including antibiotic administration (**Van der Waaij *et al*., 1982**) and as a result of infectious agents such as toxigenic *Escherichia coli*, *Salmonella enteritis* and viruses (**Silva *et al*., 1999**). Innovative approaches have been tried as alternative to antibiotics in treating gastrointestinal diseases and these include using live biotherapeutic agents such as yeast and *Lactobacillus* species (**Fuller, 1992**). Also, some people use *Lactobacillus* for general digestion problem; irritable bowel syndrome (**Niedzielin *et al*., 2001**), colic in babies (**Savino *et al.*, 2007**), crohn's disease (**Marteau *et al*., 2006**), and a serious gut problem called necrotizing enterocolitis (NEC) in new born babies (**Luoto *et al*., 2010**). It is also used for infection with *Helicobacter pylori* the type of bacteria that causes ulcers (**Sakamoto *et al*., 2001**) and for other types of infection including urinary tract infections (UTIS) and to prevent respiratory infection in children attending daycare centers. It is also used for skin disorder such as fever blisters, canker, sores, eczema (**Woo *et al*., 2010**), acne, high cholesterol, lactose intolerance and to boost the immune system (**Berggren *et al*., 2011**).
Lactobacilli have been used for decades against infectious diseases (**Bernet *et al*., 1994**) and their antimicrobial activities against pathogens have been extensively studied. These organisms have been widely used as probiotics (**Tannock, 1999**). Many of these lactic acid bacteria are known to produce antibacterial substance including bacterocin which can inhibit the growth of several pathogenic bacteria. Bacteriocin from lactic acid bacteria are natural antimicrobial peptides or small proteins with bactericidal or bacteriostatic activity against genetically related species (**Klaenhammer, 1988**). Bacteriocin can be classified broadly as those synthesized by Gram-positive and those by Gram-negative organisms, among those synthesized by Gram- positive, *Lactobacilli* bacteriocins are of commercial value (**Garneau *et al*., 2002**).
Probiotics are live microorganisms that when consumed in an adequate amount as part of the food, confer a health benefit on the host (**FAO/WHO, 2001**). An experimental focus of probiotic LAB strains has indicated that this potential might play a considerable role during in vivo interactions occurring in the human gastro intestinal tract (**Avonts and De Vuyst, 2001; Kim *et al*., 2003**). Therefore the aim of this research is to isolate and identify *Lactobacillus* species from some foods and to carry out *in vivo* assay of the bacteriocin produced by the isolated *Lactobacillus* species.

MATERIAL AND METHODS

Sample Collection

Four food samples which are palm wine, *ogi*, *kun-nu* and yoghurt were purchased from local producers in Ogbomosho, Oyo State, Nigeria.

Microbiological Analysis

One millilitre of each sample was measured, dispensed into 9 mL of sterile peptone water in McCartney bottles, and homogenized by thorough shaking. The samples were diluted into 10-fold dilutions and appropriate dilution was plated on deMann Rogosa Sharpe agar (Oxoid) to obtain LAB. Plates were incubated at 30^{O}C for 48 h under anaerobic conditions. Colonies were randomly picked from

the agar plates and the strains were streaked out repeatedly to check for purity and sub-cultured on fresh agar plates of the isolation media, followed by microscopic examinations. The stock cultures were routinely maintained on MRS agar slants kept in refrigerator at 4^{O}C. The organisms were kept in freezing medium, by inoculating pure cultures into MRS broth with 20% glycerol and stored at -20^{O}C.

Phenotypic Characterization

The cell morphology of the presumptive LAB strains were viewed by using a phase contrast microscope (Olympus CH3-BH-PC, Japan) after Gram staining and testing for catalase activity. Strains were preliminarily identified based on phenotypic properties such as the ammonia (NH_3) production from arginine, growth at 15^{O}C and 45^{O}C, ability to grow at 6.5% of sodium chloride (NaCl), hydrogen peroxide production, pH reduction in MRS broth, and gas (CO_2) production from glucose, according to **Dykes (1995)**. All the strains were tested for the sugar fermentation patterns.

Identification of Strains

Strains identification was carried out according to physiological and biochemical characteristics, as described by **Schleifer and Kilpper-Balz (1984)**. To confirm the identity of the isolates, total genomic DNA was extracted using the method described by **Oladipo *et al.* (2013)**. Identification was carried out by sequencing of the 16S rRNA genes using the primers designated as FD1 (5'-AGAGTT TGATCCTGGCTCAG - 3') forward and RD1 (5'-AAGGAGGTGATCCAGCC-3') for reverse (**Weisburg *et al*., 1991**).

Determination of bacteriocin production and antimicrobial spectra

This was carried out using the modified method of **Oladipo *et al.* (2014a, b; 2015a**). Briefly, *Lactobacillus species* were propagated in 100mL MRS broth for 72 hours at 30^{0}C anaerobically. For extraction of bacteriocin, a cell-free solution was obtained by centrifuging the culture (6708 g for 20 minutes at 4^{0}Cwith Beckman L5050B) and was adjusted to pH 7.0 by means of 1M NaOH to exclude the antimicrobial effect of organic acid. Inhibitory activity from hydrogen peroxide was eliminated by the addition of 5mg/mL catalase and filter sterilized through 0.22 mm filters. The antimicrobial activities of the supernatants were determined by well diffusion assay, 10µL aliquots of supernatants were placed in wells (3-mm diameter) cut in cooled soft LB agar plates previously seeded with indicator microorganisms. After 2 hours at 4^{O}C, the plates were incubated at 30 ^{O}C or 37^{O}C for growth of the target organism; after 24 hours, the plates were examined for growth inhibition zones (**Daba *et al*., 1991**).

Purification of Bacteriocins

The crude bacteriocin samples produced were treated with solid ammonium sulphate to 0, 30, 35, 40, 45, 50, 55, 60, 65 and 70% saturation. The mixtures were stirred for 2 hours at 4^{0}C and later centrifuged at 6708 g for 1 hour (4^{0}C). The precipitates were re-suspended in 25 ml of 0.05M potassium phosphate buffer (pH 7.0). Dialysis was followed in a tubular cellulose membrane against 2 liters of the same buffer for 18 hours in spectrapors No. 4 dialysis tubing. Assay of the bacteriocin activity was carried out (**Rammelsberg and Radler, 1990**). Agar well diffusion assay procedure was used, aliquots of 50µL from each bacteriocin dilution were placed in wells in plates seeded with the bioassay strain. The plates were incubated overnight at 30^{0}C and the diameters of the inhibition zones were taken.

***In vivo* Assay**

Infection and Treatment

The weights of all the animals were recorded prior to infection and all rats were confirmed to be healthy. The animals were randomly divided into 8 groups of 10 animals each. The rats in Group 1, the control were not infected but received normal saline (0.4µL) while the other seven groups were infected by oral administration with 0.1ml of *E. coli* (5.7×10^{7} CFU/mL). Group 2 was infected but received no bacteriocin, Groups 3 to 8 animals were given 1280 AU/mL of the bacteriocin from *L. plantarum* MO21, *L. plantarum* MP12, *L. casei* MK21, *L. casei* MO11, *L. brevis* MK11 and *L. buchneri* MY21 respectively. This treatment was administered twice in a week for 3 weeks (**Oladipo *et al*., 2014a**).

Clinical Examination

A modification of the method of **Oladipo *et al*. (2014a; 2015b**) was used for clinical examination of the experimental animals. The animals were weighed on a daily basis; blood samples were drawn from all the animals after three weeks of treatment and the blood was allowed to cloth at room temperature and then centrifuged at 310 g for 12 minutes. The serum was collected and used for analysis, which included total protein, blood glucose, albumin, globulin, and enzymes activities (AST and ALT).

Serum Biochemical parameters Analysis

Glucose Analysis: The quantitative determination of serum glucose was carried out using commercially available diagnostic experimental kits purchased from Diagnosticum Limited (Budapest, Hungary).

Albumin Assay: Quantitative colorimetric albumin determination was carried out by using Albumin Assay kit which was purchased from BioSino Biotechnology & Science Inc. (China). Serum total protein was determined according to Lowry's method. Total protein minus albumin equals globulin.

Transaminases Assay: The determination of AST and ALT was based on the fact that phenyl hydrazone which was produced after incubating the substrate with the enzyme was measured spectrophotometrically. The amount of phenyl hydrazone formed was directly proportional to the enzyme quantity. Stanadard kits for the determination of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were obtained from Span diagnostics, Surat (Gujurat). Colorimetric procedure in which the oxaloacetate and/or pyruvate formed in either the AST or ALT reaction is combined with 2, 4-dinitrophenylhydrazine to yield a brown-coloured hydrazone which is measured at 505 nm.

Histological assessment of the gastric tissues and GIT

The animals were anesthetized by ketamine (12 mg kg^{-1} body weight) followed by cervical dislocation for killing, stomachs and small intestines were removed and fixed in 10% formalin and embedded in paraffin. The sections (5µm) were cut with a microtome, stained with hematoxylin and eosin, and assessed under an Olympus microscope (Olympus Optical Co., GMBH, Hamburg, Germany). Images were captured using Camedia software (E20P 5.0 Megapixel; Hamburg, Germany) at 20X magnification (**Oladipo *et al*., 2014a; 2015b)**.

Statistical analysis

Statistical analysis of the data obtained was carried out using GraphPad Instat 3 software. Comparison between groups was made using one-way analysis of variance.

RESULTS AND DISCUSSION

Identification of bacterial strains

The isolates were found to be Gram's positive rod, catalase, oxidase, methyl red, coagulase, urease and indole negative. They were unable to hydrolyze casein and gelatin but they were all able to grow at pH of 3.9 and 9.2. In this present study, six organisms isolated from food samples (*ogi*, *kunnu*, yogurt and palm wine) were characterized and identified to be *Lactobacillus* species using polyphasic taxonomy approach as described by **Oladipo *et al.* (2013)**. The sequences were deposited in the GenBank database and accession numbers assigned to each strain as shown in Table 1.

Determination of bacteriocin production and antimicrobial spectra

Determination of the antimicrobial activity was carried out for all the *Lactobacillus* isolates and the bacteriocin produced showed antimicrobial inhibitory activity against *Serratia marcescens, Micrococcus luteus, Bacillus cereus, Bacillus subtilis, Escherichia coli, Lactococcus lactis, Staphylococcus aureus and Bacillus pumilus* but no activity was shown against *Proteus mirabilis, Proteus vulgaris, Klebsiella pneumonia, Enterobacter cloacae and Pseudomonas aeruginosa. Lactobacillus plantarum* MO21 and *Lactobacillus plantarum* MP12 had broad spectra of activities against the indicator organisms used (Table 2). The culture supernatant of *Lactobacillus* species was found to posses' antimicrobial activity against Gram positive and Gram negative bacteria. This supports the previous findings of **Nes *et al.* (1996)** who reported that bacteriocins are proteinaceous compounds with inhibitory activity against more or less related bacterial genera. **Juven *et al.* (1992)** also reported that the ability of the *Lactobacilli* to produce metabolites such as bacteriocins has been suggested as being responsible for their ability to inhibit other bacteria.

In-vivo evaluation of the effects of the bacteriocinogenic strains and their bacteriocins

Table 3 shows the result of weight gained by the experimental rats during *in-vivo* assay. No death was recorded and their weights were measured on a weekly basis for three weeks. The control had the highest weight gained after the first, second and third week while the infected but not treated group showed the lowest weight gained. No changes in rats' behavior, daily activity or physiology of the

experimental rats was observed and the weight gained was regular. The weight gain result reveals that the body weight of the rats increased during the 3weeks of experiment. The low gained weight recorded in the untreated rats may be as a result of the infection induced by *E. coli* while the high gained weight recorded in the bacteriocin treated rats confirmed the findings of **Fuller and Gibson (1997)** who reported that bacteriocin has been used as growth promoters due to their ability to suppress the growth and activities of growth depressing micro flora and their ability in enhancing absorption of nutrients through the production of digestive enzymes.

Biochemical evaluation of sera

The result of the biochemical analysis showed an increase in the values of AST, ALT, albumin, total protein, globulin, cholesterol, bilirubin and glucose of the untreated rats due to the prolong infection caused by *E. coli.* But the bacteriocin treatment normalized the blood serum of the treated groups (Table 4). With regards to serum biochemical analysis, the high level of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) of the untreated group is an indication of liver damage or dysfunction caused by the administration of *E. coli.* Alanine aminotransferase (ALT) is principally found in the liver and is regarded as being more specific than AST for detecting liver cell damage (**Johnston, 1999**; **Cheesborough, 1991**). A rise in ALP activities has been linked with an increased osteoblastic activity (**Baron *et al.* 1994**) and lack of bile flow (cholestasis). **Devaraj (2012)** reported that when body tissue or an organ such as the heart or liver is diseased or damaged additional AST is released into the bloodstream. The lower AST and ALT values in rats treated with bacteriocin indicate liver function improvement brought about by bacteriocin treatment. As these parameters represent liver function, increase in their levels will indicate liver damage (**Gad, 2007**). Similar positive effect on the biochemical parameters of rats' serum were also observed by other authors with different probiotic treatment (**Fukushima and Nakano, 1995**).

Biochemical analysis showed high total protein, albumin, globulin, glucose, cholesterol and bilirubin in the rats from untreated group compared to the control and treated rats. **Amdekar *et al.* (2010)** reported that *E. coli* infection can cause the rise in total protein of blood serum and **Koneko (1989)**, reported that increase in total protein and globulin in serum have been associated with bacteria septicaemia and liver disease. The observed elevated bilirubin levels in the blood of infected rats may arise from free radical damage caused by the pathogen. This damage may be to the liver, red blood cells or the heart. **Baranano *et al.* (2002)** reported that elevated bilirubin levels in blood shows risk of cardiovascular diseases, increased breakdown of red blood cells and liver failure. **Koneko (1989)** reported that increase in total protein and globulin concentration in serum has been associated with bacterial septicemia and liver disease which was also observed in untreated rats. The lower Cholesterol level in the treated group when compared to untreated rats confirmed the ability of bacteriocin to function as anticholesterol substance. **Casas and Dobrogosz (2000)** said generally that *Lactobacilli* have anticholesterolaemic effect and **Bertazzoni *et al.* (2001)** found that *Lactobacilli* have direct effect on cholesterol levels by assimilation and removal from the growth medium.

Histological assessment of gastric tissues and GIT

The histopathology of the stomach showed that the *E. coli* infection caused disruption at the junction between the sub mucosal and muscular layer, infiltration by inflammatory cells but this was normalized by bacteriocin treatment as no visible lesions was observed in the stomach of the bacteriocin treated rats (Fig. 1). Also, the histopathology of the intestine of the untreated rats showed blunted and disintegrated villi tips and there were large parasitic sections along with little mucosal debris in the intestinal lumen which was cured by the bacteriocin treatment (Fig. 2). The result of the histopathology of the stomach of rats after three weeks of bacteriocin treatment showed no visible lesion as compared to the control. Also, the mucosa, submucosa and muscular layer were unaffected. The protection of the gastro intestinal tract was observed in the intestine of the bacteriocin treated rats because no visible lesions was observed and the serosa, villi and lumen was intact after three weeks of bacteriocin treatment. This indicated that bacteriocin treatment was able to cure the infection caused by the *E. coli* and this is also in accordance with **Oyetayo *et al.* (2003)** whose histopathological result also confirmed the protective effect of the *Lactobacillus*. There has been a number of studies that reveal the probiotic potential of *Lactobacilli* as health promoting bacteria in man and animals (**FAO/WHO, 2001**). They play a major role in protecting the immune system against pathogens residing in the human body.

Table 1 *Lactobacillus* species used in this study

Name of isolates	Source of isolation	Accession number
Lactobacillus plantarum MO21	*Ogi*	KJ739519
Lactobacillus plantarum MP12	*Palm wine*	KJ739520
Lactobacillus casei MK21	*Kunnu*	KJ739521
Lactobacillus casei MO11	*Ogi*	KJ739522
Lactobacillus brevis MK11	*Kunnu*	KJ739523
Lactobacillus buchneri MY21	*Yoghurt*	KJ739524

Accession number were assigned by GenBank of National Centre for Biotechnological Information (www.ncbi.nlm.nih.gov/genbank/)

Table 2 Antimicrobial activity of *Lactobacillus* species against indicator organisms

Indicator organisms	*L. plantarum* MO21	*L. plantarum* MP12	*L. casei* MK21	*L. casei* MO11	*L. brevis* MK11	*L. buchneri* MY21
Serratia marcescens	10.1± 0.21	11.0± 0.22	10.0± 0.11	9.0± 0.23	10.0± 0.12	9.5± 0.13
Micrococcus luteus	13.0± 0.23	12.0± 0.31	11.0± 0.31	10.0± 0.14	11.0± 0.14	8.0± 0.24
Proteus mirabilis	-	-	-	-	-	-
Proteus vulgaris	-	-	-	-	-	-
Bacillus cereus	10.0± 0.30	11.0± 0.21	9.0± 0.14	11.0± 0.12	9.0± 0.23	10.0± 0.31
Bacillus subtilis	11.5± 0.25	10.5± 0.11	8.0± 0.13	12.0± 0.23	10.0± 0.15	11.0± 0.23
Klebsiella pneumoniae	-	-	-	-	-	-
Escherichia coli	12.0± 0.10	11.5± 0.10	9.0± 0.11	9.0± 0.15	11.0± 0.16	8.5± 0.12
Shigella flexneri	12.5± 0.12	12.0± 0.14	-	-	-	-
Lactococcus lactis	11.0± 0.11	10.5± 0.13	10.0± 0.25	8.0± 0.31	8.5± 0.11	12.0± 0.24
Enterobacter cloacae	-	-	-	-	-	-
Staphylococcus aureus	13.0± 0.20	12.0± 0.12	11.0± 0.12	9.5± 0.16	9.5± 0.10	10.0± 0.20
Pseudomonas aeruginosa	-	-	-	-	-	-
Aeromonas hydrophilia	9.0± 0.21	9.2± 0.22	-	-	-	-
Salmonella typhimurium	10.0± 0.21	10.0± 0.23	9.0± 0.24	8.7± 0.22	-	-
Bacillus pumilus	11.0± 0.22	10.5± 0.25	8.0± 0.26	8.5± 0.24	10.0± 0.24	13.0± 0.11

Each value is a mean of 3 replicates ± standard deviation, - = Not Detected, diameter of zones of inhibition are in millimeter

Table 3 Weight gained by experimental rats during experimental period

Weeks of treatment	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
Week 0	150± 0.47	150± 0.41	150± 9.81	150± 9.56	150± 0.47	150± 9.40	150± 0.47	150± 9.47
Week 1	158± 0.29	150± 0.22	152± 0.26	151± 0.22	151± 0.22	152± 0.26	152± 0.27	151± 0.26
Week 2	187± 1.41	153± 0.90	170± 1.47	171± 0.99	170± 0.79	173± 1.20	172± 1.00	173± 1.01
Week 3	200± 1.21	158± 0.89	190± 0.81	194± 0.92	196± 0.91	194± 0.99	192± 0.96	193± 1.09

Each value is a mean of 3 replicates ± standard deviation, weight of experimental animals are in gram

Table 4 Biochemical analysis of the blood serum of experimental rats

Biochemical parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Untreated
Total protein (g/dl)	1.9± 0.21	4.9± 0.41	3.9± 0.41	3.9± 0.21	3.9± 0.41	3.9± 0.51	3.9± 0.25	3.9± 0.11	4.9± 0.41
Glucose (g/dl)	119.6± 24.99	132.9± 20.02	130.0± 15.56	130.9± 20.02	130.9± 20.02	130.2± 15.86	130.4± 27.97	130.2± 15.56	132.9± 20.02
Globulin (g/dl)	0.9± 0.41	1.2± 0.49	1.04± 0.47	0.9± 0.21	1.0± 0.49	0.9± 0.21	1.0± 0.50	1.0± 0.17	1.2± 0.49
Cholesterol (g/dl)	69.7± 8.71	78.0± 9.61	70.3± 9.81	70.3± 9.81	70.3± 9.81	70.3± 9.99	71.0± 9.60	59.9± 5.49	78.0± 9.61
Bilirubin (g/dl)	0.45± 0.94	0.72± 0.10	0.49± 0.09	0.63± 0.10	0.69± 0.18	0.62± 0.21	0.67± 0.04	0.62± 0.10	0.72± 0.10
ALT (IU/l)	55.0± 8.30	71.0± 23.00	62.0± 29.00	67.0± 16.00	60.0± 27.00	63.0± 14.00	63.0± 23.00	49.0± 9.30	71.0± 23.00
AST (IU/l)	280.1± 20.49	304.6± 45.40	280.1± 40.31	292.8± 23.20	292.1± 23.20	292.8± 23.20	292.1± 22.31	282.2± 20.49	304.6± 45.40
Albumin (g/dl)	2.8± 0.29	4.0± 0.11	3.0± 0.11	3.0± 0.07	3.0± 0.11	3.0± 0.07	3.0± 0.03	3.0± 0.24	4.0± 0.11

Each value is a mean of 3 replicates ± standard deviation

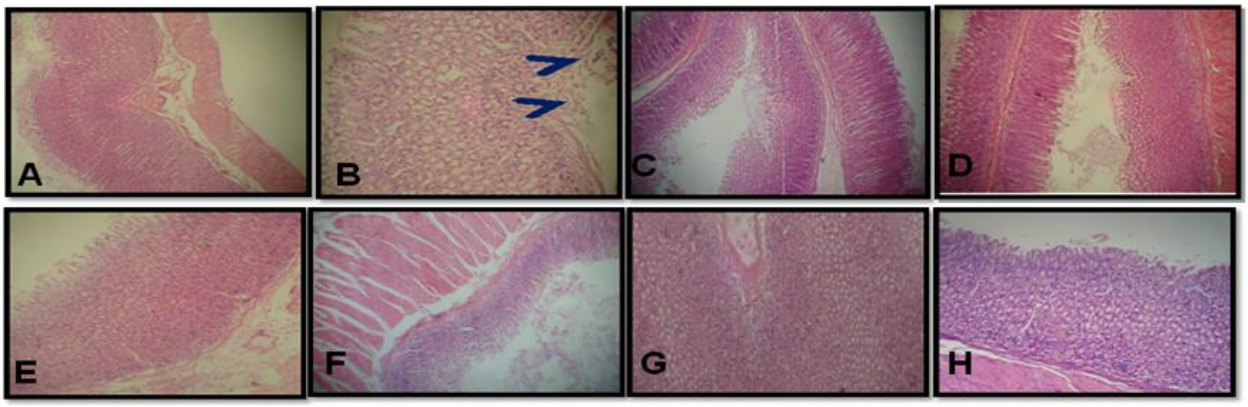

Figure 1 Histology of rat gastric tissues after infection with *E. coli* and treatment with bacteriocin.
A - H = Group1 – Group 8

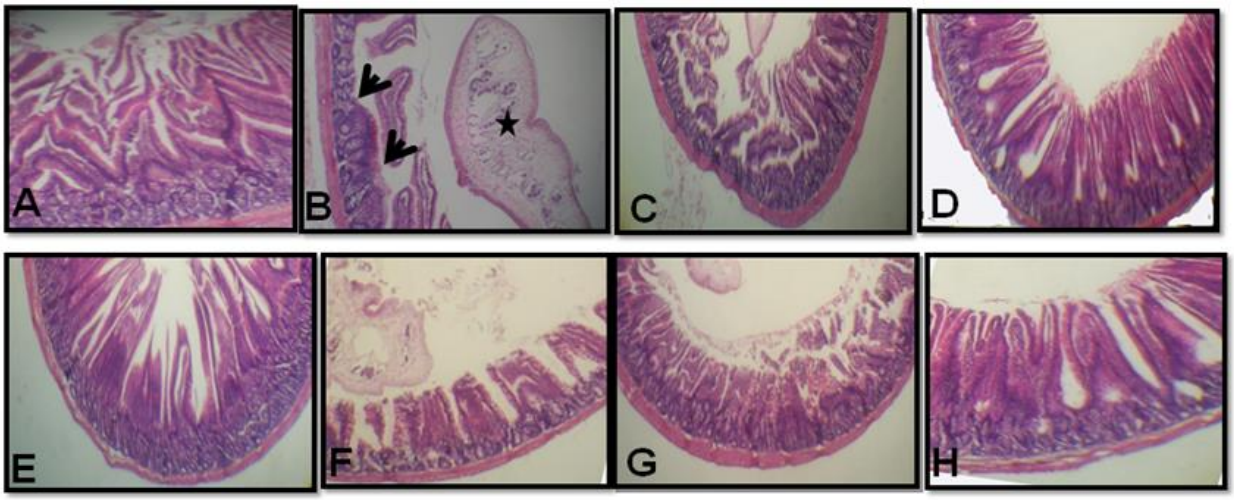

Figure 2 Histology of the small intestine of experimental rats after infection with *E. coli* and treatment with bacteriocin.
A - H = Group1 – Group 8

CONCLUSION

Conclusively, the antimicrobial characteristics of the *Lactobacillus* species has a positive impact on its use as starter culture for traditionally fermented foods with a view of improving the hygiene and safety of the food products so produced and the bacteriocin produced is responsible for an important effect in the disruption of *E. coli* plasma membrane and protection of the gastro intestinal tract as revealed in Sprague dawley rats. Hence , the improvements of liver function, protection of gastric tissues and GIT from infection are evidences supporting the probiotic nature of the strains . Therefore, the special characteristics of these *Lactobacillus* species can positively contribute to their use as probiotics. The use of these *Lactobacillus* species and their bacteriocins may be beneficial for biomedical purposes.

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COMPARISON OF HYALURONIC ACID BIOSYNTHESIS BY THE RECOMBINANT *Escherichia Coli* STRAINS IN DIFFERENT MODE OF BIOREACTOR OPERATION

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ABSTRACT

Recombinant *E. coli* was used as a host to explore the biosynthesis of Hyaluronic acid (HA) in Gram-negative strain using the optimized medium. In addition, measurement of HA molecular weight produced by the recombinant strains in shake-flask and 2 L stirred tank bioreactor (batch and fed-batch cultivation) were compared too .In this study, the *hasA* and *hasE* genes expressed together in a plasmid in recombinant *E. coli* strains were successfully produced HA using glucose as a carbon source under aerobic condition. Production of 127 mg/L HA was observed in a batch fermentation process with the highest HA molecular weight (70056 Da) using strain sAE in 2 L stirred tank bioreactor with controlled DO at 30%. The fed-batch culture with constant feeding of glucose was favorable for cell growth and the highest OD_{600} achieved at 1.923. However, the HA biosynthesis and molecular weight in fed-batch culture were lower than that observed in the batch culture.

Keywords: Recombinant, Hyaluronic acid, Batch cultivation, Fed –batch cultivation

INTRODUCTION

HA can be used in various fields such as cosmetics, nutraceutical and osteoarthritis due to its biological and physiological properties **(Kogan *et al*., 2007)**. The worldwide market value is estimated to be over US $ 1 billion **(Liu *et al*., 2011)**. In past researches, a vast number of scientists argued that *Streptococci* spp. can be fairly difficult or expensive to mass cultivate and it was also found to be a challenge to genetically manipulate the strains. It was shown to have the potential to produce exotoxins, which is unsuitable for producing HA applied for medical applications **(Kogan *et al*., 2007; Shih *et al*., 2013)**. All these drawbacks may have played a part in encouraging researchers to favor the incorporation of novel biotechnological productive methods. This was to solve the pathogenic problems as well as to avoid any contaminations that may have a negative effect towards health. Thus, in the recent years, HA biosynthesis in recombinant microorganisms through metabolic engineering emerged as an attractive alternative method that could reduce safety concerns associated with the use of pathogenic microorganisms and avian products.

Appropriate process conditions are crucial for the metabolic engineered strains to reach their maximum potential, as they may allow the modified metabolic routes to behave as predicted. The first metabolic engineering study effort for recombinant biosynthesis of HA was reported by **Frohberg and Koch (2004)** who expressed the HA synthase genes of human and Chlorella virus in algal plant. There have been a few approaches for the production of HA using metabolically engineered microorganisms. Both Gram-positive and Gram negative bacteria were used as hosts in producing the HA **(Liu *et al*., 2011).** These include *Bacillus sp.* **(Widner *et al*., 2005; Chien and Lee, 2007a),** *Lactococcus lactis* **(Chien and Lee, 2007b; Prasad *et al*., 2010);** for Gram-negative bacteria were *Escherichia coli* **(Yu and Stephanopoulos, 2008; Rehm 2009)**, and *Agrobacterium* sp. **(Mao and Chen, 2007).** For instance, Gram positive *Bacillus subtilis* was used as expression host, due to its ability to secrete similar cell-wall composition features with *Streptococcus* **(Widner *et al*., 2005). Yu and Stephanopoulos (2008)** incorporated the use of recombinant *E. coli* under fed-batch fermentation using Luria broth as the fermentation medium and resulted in a HA concentration of 0.19 g/L. Another study using *E. coli* under batch fermentation using also Luria broth as the fermentation medium was able to produce a HA concentration of 0.325 g/L **(Jongsareejit *et al*., 2007).** The HA concentration produced by the recombinant *B. subtilis* indicates that the genetic engineering approach provides new opportunities for capsular polysaccharides to be produced in a heterologous host.

Uridine diphosphate (UDP)-glucose (UDP-GlcA) and UDP-GlcNAc are two nucleotide sugar substrates required for HA synthesis and are produced as precursors of bacterial cell wall components. In addition to that, there is stiff competition for metabolites between HA synthesis and cellular growth due to glucose-6-phosphate and fructose-6-phosphate being consumed in the pentose phosphate pathway and glycolysis respectively **(Mao *et al*., 2009).** It has been elucidated that HA is synthesized in various cells by polymerization of the monosaccharides from two nucleotide sugar (UDP)-glucuronic acid (UDP-GlcUA) and UDP-N-GlcNAc via HA synthase into a long chain **(DeAngelis *et al*., 1993)**. As mentioned earlier, UDP-GlcNAc is commonly present in most bacteria as precursors of cell wall components. In contrast, UDP-GlcUA is not commonly used for building their cell wall **(Chien and Lee, 2007a).** Therefore, HA synthase is the only enzyme that lacking in most bacteria important for HA biosynthesis.

In this study, recombinant *E. coli* was used as a host to explore the biosynthesis of HA in Gram-negative strain using the optimized medium. In addition, measurement of HA molecular weight produced by the recombinant strains in shake-flask and 2 L stirred tank bioreactor (batch and fed-batch cultivation) were compared.

MATERIALS AND METHODS

Microorganism and Medium

Recombinant *E. coli* Rosetta (DE3) (CalbioChem, Germany) was used as an expression host of the pRSF and duet PCDF plasmids. The gene sets included two genes set *hasA* and *hasE* genes (strain sAE) in one plasmid and *hasA* and *hasE* in one plasmid with addition of *hasE* gene in separate plasmid (strain sAE-E). The cells were grown at 37ºC with 200 rev/min shaking speed for 12 h either in Luria-Bertani (LB) plates or in the LB liquid medium (as an inoculum) supplemented with kanamycin (CalbioChem, Germany) (50 µg/mL) and chloramphenicol (BioWorld, USA) (35 µg/mL) for sAE strains, additional 50 µg/mL of streptomycin (CalbioChem, Germany) for sAE-E strains. The fermentation medium contained (in g/L) glucose 50, tryptone 15, yeast extract 5, KH_2PO_4 2, K_2HPO_4 2, $MgSO_4.7H_2O$ 0.5. The culture medium was sterilized at 121ºC for 15 min. The glucose and $MgSO_4.7H_2O$ were autoclaved separately.

The kanamycin, chloramphenicol and streptomycin were sterilized by filtration using 0.45 µm nylon syringe membrane filter (ThermoScientific, USA). Fermentations were carried out in duplicates and the mean value of each experiment was obtained.

Fermentation in Shake-flask

To investigate the effect of incubation temperature (30ºC, 37ºC and temperature-shift, 37ºC-30ºC) for HA biosynthesis by the recombinant strains (sAE and sAE-E), experiments were carried out using a 250 mL Erlenmeyer flask. The flask cultures were incubated in rotary shaker at 200 rev/min with different temperatures. To initiate the fermentation, 1 loop of colony culture was transferred to 50 mL of LB inoculum medium for 12 hours at pH 7, and 10 % (v/v) of inoculum was inoculated into the 250 mL shake flask containing 50 mL of the production medium and run for another 12 hours at initial pH 7 with different temperatures. For temperature shift, temperature was shifted to 30ºC once inducer is added. Inducer of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce the expression of *hasA* genes when OD_{600} reached about 0.6.
Two different induction times (when cell growth OD_{600} = 0.6; OD_{600} = 1.0) were investigated by using 0.5 mM of IPTG. Four different IPTG concentrations (0, 0.1, 0.5, and 1.0 mM) were investigated when the cell growth reached the middle exponential phase. All experiments are run at 37ºC, 200 rev/min and with initial pH 7.

Fermentation in 2 L Stirred Tank Bioreactor

Batch Fermentation

All experiments were conducted using a 2 L stirred-tank bioreactor using strains sAE and sAE-E. The dissolved oxygen of batch HA fermentation was controlled according to requirement of the experiments (20%, 30% and 50%). Dissolved oxygen was provided by sparging filtered air at a flow rate of 1 vvm and was maintained according to requirement by automatically adjusting the agitation rate from 50 to 350 rev/min with half-pitched double blade helical ribbon impeller, via sequential cascade control.

Fed-batch Fermentation

Fed-batch fermentations were carried out in a 2 L stirred-tank bioreactor equipped with half-pitched double blade helical ribbon impeller. The working volume was 1 L with initial batch culture working volume 500 mL. The fed-batch feed protocol was designed as initially the cells were cultivated in 4 h of batch phase, to stabilise the cells and control the lag phase behaviour. The cultivation was the batch phase followed by a constant glucose feed before glucose was depleted. Initial glucose (10 g/L) was added to create batch phase before the constant feed phase was started. The feed solution (10 g/L glucose and other components of medium) were pumped into the bioreactor at a feeding rate of 2 ml/min by a peristaltic pump (Watson Marlow 101 U/R, England). The fermentation was carried out for 12 h, with temperature maintained at 37ºC and pH controlled at 7.0 by the automatic addition of 3 M NaOH and 3 M HCl. A polarographic oxygen electrode was used to determine the dissolved oxygen level (DOT). The dissolved oxygen never dropped below 30% air saturation. Also, 1 mM of IPTG was added to express *hasA* gene in *E. coli*.

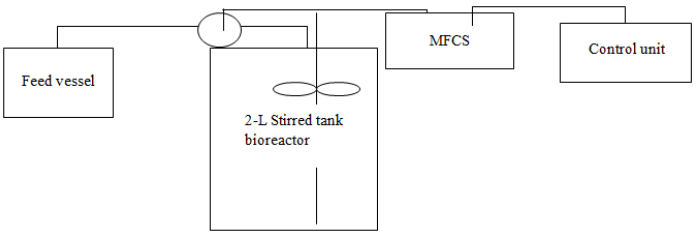

Figure 1 Schematic diagram for fed-batch fermentation set-up

The bioreactor was connected to the multifermenter control system (MFCS) for controlling the feeding rate of the substrate and also controls some of the bioreactor operating variables. Control of the peristaltic pump was facilitated using MFCS/win software, which is a fermenter supervisory control and data acquisition system (SCADA) for simultaneous control of multiple fermenters.

Analytical Methods

All samples were withdrawn at regular time intervals for analysis of cell, HA and glucose concentration. Cell growth was observed by measuring the optical density of the culture broth at 600 nm using a spectrophotometer. Correlation between the dry cell weight (DCW) and OD was estimated from several batch experiments using the equation: DCW (g/L) = 1.489 x OD

The supernatants were used for HA and glucose determination. After the removal of the cell pellet for cell growth determination, two volumes of ethanol were added to one volume of the supernatant in a 15 ml centrifuge tube and the solution was refrigerated at 4°C for 1 h to precipitate HA. The precipitate was collected by centrifugation at 3,000 x g for 20 min and was re-dissolved with a 2 to 3-fold volume of distilled water. The HA concentration was determined using the carbazole method (**Bitter and Muir, 1962**) and the optical density was measured at 530 nm. The HA concentration was calculated using a standard curve prepared at different concentrations of HA standards (Sigma-Aldrich, Malaysia). The HA molecular weight (M_w) was analyzed by high performance liquid chromatography equipped with a cation-exchange column (Aminex HPX-87H, BioRad Labs, Hercules, CA, USA), a UV detector and the mobile phase was 4 mM H_2SO_4 at a steady flow rate 0.5 ml/min.

RESULTS AND DISCUSSION

Fermentation in Shake-flask

Effect of Temperature

The ability of 2 different types of *E. coli* strains that were cloned with *hasA* or *hasE* gene from *S. zooepidemicus* ATCC 39920 **(Samsudin *et al*., 2011)** for HA biosynthesis were investigated. The HA biosynthesis capability of the metabolically engineered *E. coli* strains were investigated in shake-flask culture with different temperature in the presence of IPTG. At three different temperatures investigated (30ºC, 37ºC and 37ºC-30ºC), a distinct stationary phase was observed following the exponential phase. As seen from **Table 1**, the cell production for all strains had very little deviance which was in the range of OD_{600} between 0.8 to 1.2. The IPTG inducer was added at 2 h of fermentation when OD_{600} reached to about 0.6 and the cell growth was inhibited once IPTG was added and decreased from 1.1 to 0.6 after 12 h fermentation (data not shown). This indicates that the host strain is susceptible to IPTG **(Chien and Lee, 2007a).** All the recombinant strains were grown better when the temperature shift from 37ºC to 30ºC once the IPTG was added; in contrast, cell growth was the lowest at 37ºC in comparison to cell grown at 30ºC. The HA biosynthesis is the highest at 37ºC for all strains, indicating cell lysis at this temperature (data not shown). However, little cell lysis, based on the HA biosynthesis in medium, was observed at 30ºC and temperature shift from 37ºC to 30ºC.
Cell growth inhibition was observed during HA accumulation. Similar phenomena were observed with all recombinant strains investigated. As can be seen from **Table 1**, the cell production was similar for sAE and sAE-E cultures. However, the HA production was consistently at least 2-fold and above higher in the strain sAE culture compared to sAE-E cultures. **Yu and Stephanopoulos, (2008)** deduced that gene coding for phosphoglucoisomerase (pgi, corresponding to *hasE* in HA synthesis operon) probably plays a critical role to obtain a higher production of HA.
An appreciable amount of HA (<110 mg/L) was observed for all strains before IPTG induction. The observed HA should result from the expression of HA synthase, due to the optimized medium employed for cell cultivation does contain 50 g/L glucose, the HA detected before IPTG induction was probably resulted from the glucose-induced expression of HA synthase. At 30ºC fermentation, sAE-E produced 62.7 mg/L HA, and the highest HA produced is by sAE (86.8 mg/L). On the other hand, the maximum HA biosynthesis was observed when all fermentation run under 37ºC. The sAE yielded about twice the amount of HA (109.6 mg/L) in shake-flask culture **(Table 1).** However, for strains sAE-E, the HA biosynthesis was remained low. It is possible that the two plasmids applied were not stable enough for *has* gene to express. In order to express two heterologous enzymes simultaneously, the energy burden not only slows down the cell growth but also reduces the expression of these two enzymes **(Chien and Lee, 2007b)**. The successful expression of these two heterologous enzymes demonstrates that PRSF vector constructed is effective for protein expression.

Table 1 Effect of temperature on cell growth and HA biosynthesis by different recombinant *E. coli* strains in shake-flask culture after 12 hours of fermentation

Temperature	30ºC	37ºC	37ºC-30ºC
sAE strain			
OD_{600} (nm)	0.964	0.849	1.043
HA (mg/L)	86.8	109.6	74.8
MW HA (Da)	11021	26802	9872
sAE-E strain			
OD_{600} (nm)	0.972	0.877	0.989
HA (mg/L)	62.7	28.7	30.8
MW HA (Da)	11339	27344	10145

With co-expression of a heterologous phosphoglucoisomerase (sAE), as consequence, the HA concentration was enhanced from 75.7 mg/L to 109.6 mg/L. The results were comparable to sseA and sseAB strains developed by **Yu and Stephanopoulos, (2008)** which are 27.8 and 203.5 mg/L HA, respectively. It is interesting to note that over-expressing of *hasA* alone does not seem to hinder

the cell growth and the *hasA* is not titrating away sugars that are required for cell growth, at least not to dangerously low levels. The over-expression of *hasA* caused a significant increase in HA yield and also significantly lowered the molecular weight of HA. The low molecular weight may be clarified by the increased competition for a foxed pool of UDP- monomers leading to sub-optimal levels of UDP-Glc UA and UDP-GlcNAc **(Chen *et al*., 2009).**

Effect of Inducer on sAE Growth

The sAE strain was selected to grow on the optimum medium with glucose as a sole carbon source incubated at 37ºC. The growth curve of the sAE strain cultivated in the presence and absence of IPTG **(Figure 2)** clearly shows that once the inducer IPTG is added, it will cause the cell growth inhibition. In contrast, the cell grew well in without IPTG addition fermentation. The IPTG inducer was added at 2 h of fermentation when OD_{600} reached about 0.6. Both growth patterns of cells were same before IPTG is added, the cell growth started to maintain once IPTG is added after 2 h, however, when the fermentation was carried out without induction, the cell growth was still increased exponentially until 10 h. The cell could grow to 1.113 OD_{600} in the absence of IPTG. In contrast, in the presence of IPTG, cell growth was inhibited the cell decreased from 1.113 to 0.748 OD_{600} after 12 h cultivation. This indicates the strain sAE is susceptible to IPTG. The advantage of IPTG for in vivo studies is that it cannot be metabolized by *E. coli*, therefore the growth rate of cells (usually maintained with glycerol as the carbon and energy source), is not a variable in the experiment and since cells do not metabolize IPTG, its concentration does not change during the course of an experiment. Besides IPTG, **Yu and Stephanopoulos (2008)**, used *L*-Arabinose as inducer to induce the expression of HAS genes in recombinant *E. coli*. Lactose has been used as a cheap alternative to IPTG to induce *lac*, but lactose has a much lower induction potential **(Kweon *et al*., 2001)** and is consumed whereas IPTG is not consumed.

Although *E. coli* is a gram-negative bacteria with the cell wall structure composed of lipopolysaccharide, peptidoglycan and lipoprotein, its metabolic pathway for HA synthesis is very similar to that of *Streptococcus* spp.; the only enzyme of *E. coli* lacks is the hyaluronan synthase. Double expression of *hasA* with *hasE* gene in a single plasmid in the recombinant *E. coli* (sAE) significantly improved the HA biosynthesis from 21.2 mg/L at 10 h (without IPTG induction) to 110.5 mg/L (with IPTG induction) at 4 h of fermentation in shake-flask culture. This indicated that the IPTG as an inducer is required to induce *hasA* and *hasE* genes. This result was significantly higher than **Yu and Stephanopoulos, (2008)** who only managed to produce 95.9 mg/L HA by using triple expression sp ABC recombinant *E. coli* strain.

The hyaluronan synthase enzyme involved in HA synthesis in *Streptococcus* and mammals has shown to prefer magnesium ions while Chorella virus HASs prefers manganese ions to stimulate the synthesis HA **(Yamada and Kawasaki, 2005).** The use of magnesium sulphate in HA biosynthesis likewise, has been preferred by recombinant *E. coli*. Large number of ATP molecules are consumed in various enzymatic reactions involved in HA biosynthesis pathway, hence source of phosphate is essential to synthesize ATPs. In this study, salt dipotassium hydrogen and potassium dihygrogen phosphate used as a source of inorganic phosphate in recombinant *E. coli* fermentation.

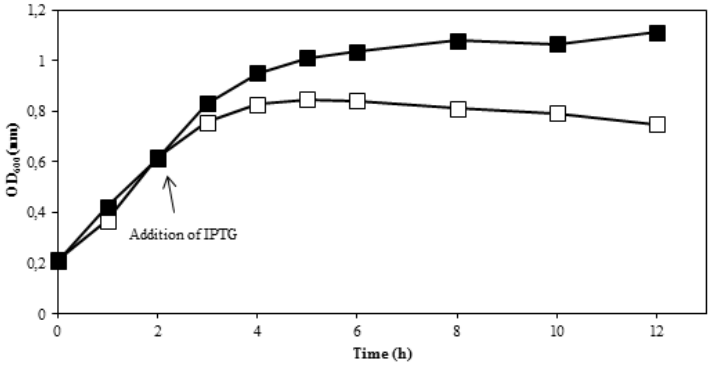

Figure 2 The growth curves of sAE strain cultivated in the shake-flask culture with the presence and absence of IPTG incubated at 37ºC. (■) – without IPTG addition ; (□) – with IPTG addition

Effect of IPTG Induction Time on sAE Growth

In order to determine the time course of induction, two shake-flask cultures experiments were performed. The first culture was grown at 37 ºC until the sAE growth, OD_{600} reached 0.6 and then 0.5 mM IPTG was added to the medium. The same amount of IPTG was also added to the second culture experiment when its OD_{600} reached 1.0. After the addition of IPTG to the cultures, the incubation was continued and samples were taken every hour to determine the cell growth, glucose consumption and HA concentration. As a result, the growth curve is shown in **Figure 3**. The cell growth was higher (OD_{600} is 1.088) with the addition of 0.5 mM IPTG at OD = 1.0 if compared to OD = 0.6. The maximum of HA biosynthesis was found to be attained 4 h after the addition of IPTG to the medium, which is 115.4 mg/L (OD=0.6) and 90.8 mg/L (OD=1.0). The induction times are considered to be important parameters in the production of the recombinant strains and they need to be carefully optimized to increase the yield of the product. The addition of IPTG in the middle of exponential phase able to achieve the higher HA production compared to addition of IPTG at late exponential phase. The strain starts goes into the stationary phase. Induction in the stationary phase reduces culture viability and can lead to production of proteases that can breakdown the desired recombinant protein **(Corchero *et al*., 2001).** On the other hand, if induction too early can slow down the doubling time of bacterial cells. The timing of induction of new recombinants need to be empirically determined for each new clone, due to the cellular responses depend on a number of interacting factors including the host/vector system **(Cserjan-Puschmann *et al*., 1999).**

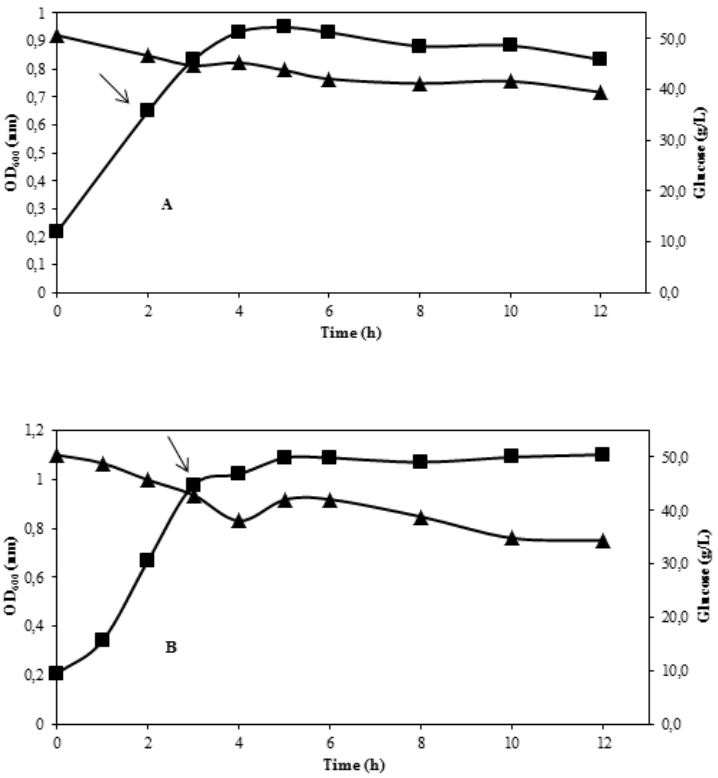

Figure 3 The growth curves of the strain sAE cultivated in the presence of IPTG with different induction time. The arrow indicated that IPTG is added. (A) IPTG added when OD_{600} is 0.6; (B) IPTG added when OD_{600} is 1.0

Effect of IPTG Concentration on HA biosynthesis by sAE

In order to determine the effect of different concentrations of IPTG on HA biosynthesis, addition of 0.1, 0.5 and 1 mM IPTG were examined. All cultures of strain sAE were grown until the OD_{600} reached 0.6 in the middle of exponential phase, 0.1, 0.5 and 1 mM IPTG was added to each culture flask respectively, and incubated till 12 hours. A control experiment was also performed in which the addition of IPTG was omitted. From **Table 2**, it can be seen that in the control experiment, the cells also grew well, however there is no HA production. The IPTG induction resulted in growth of sAE and HA production. An increment of IPTG concentration has meaningful effect on cell and HA production.

From **Table 2**, the cell growth was higher with no IPTG addition compared to 0.1 mM of IPTG addition (OD =1.113 and 1.1, respectively). The cell growth was reduced when the IPTG concentration was increased from 0.1 to 0.5 (OD= 0.949) and 1.0 mM (OD= 0.849). On the other hand, the HA concentration was the highest when the cell growth was at the lowest when 1 mM IPTG was added. This result proved the statement of there is a stiff competition for metabolites between HA production and cellular growth **(Mao *et al*., 2009).** From the **Table 2**, the HA concentration obtained are comparable, however, there is a significant difference when we compare the molecular weight of HA measured after the fermentations. Although HA with a relatively low molecular weight is broadly used in cosmetics fields, high molecular weight HA is more appealing to be used in medical applications (**Liu *et al*., 2008**). High molecular weight HA is also highly viscous which makes it ideal for some uses such as reducing the adhesion formation of post-surgery **(Takeda *et al*. 2011)**. The highest HA molecular weight can be achieved at 40928 Dalton using 1mM of IPTG, which is about 3 folds higher than other IPTG concentration. At a concentration of 1 mM the *lac* promoter appeared to be fully induced. The concentration of IPTG (between 0.1

mM and 2mM) required for complete induction is known to vary widely with clones **(Li *et al*., 1999; Madurawe *et al*., 2000).** The presence of excessive IPTG reduced the final cell density. These results concur with **Yee and Blance (1993)** who observed a reduced growth rate in *E. coli* when IPTG concentration exceeds 1 mM.

Table 2 Effect of IPTG concentration on maximum cell growth, HA biosynthesis and molecular weight by strain sAE using shake-flask culture

IPTG (mM)	OD_{600} (nm)	HA (mg/L)	MW HA (Da)
0	1.113	0	0
0.1	1.1	99.6	14263
0.5	0.949	103.5	14641
1	0.849	109.6	40928

Fermentation in 2 L Stirred Tank Bioreactor

Batch Cultivation

Batch bioreactor studies were conducted with both the recombinant strains (sAE and sAE-E) initially with controlled DO at 20 %. With both recombinant strains, it was observed that glucose was only consumed 8 to 9 g/L throughout the 10 h fermentation (as shown at **Table 3**). Cell using glucose as carbon source can theoretically be thought of as used for four different purposes in recombinant protein producing cells, i.e. for: growth, maintenance, respiration and product formation. If the concentration is very high glucose will further be used for formation of overflow metabolite formation. Accumulation of acetate is a common obstacle to achieving high levels of recombinant protein and other fermentation products in *E. coli* **(Chen and Bailey, 1993).** With excess glucose in the medium, *E. coli* cells tend to synthesize high levels of acetate and other organic acids, such as lactic acid, usually resulting in cell growth inhibition and reduced HA production due to the reduced pH of the medium **(Liu *et al*., 2011).**

The growth curve of recombinant *E. coli* strains is shown in **Figure 4**. The cell growth curve in sAE and sAE-E cultures are same, the maximum OD_{600} in strain sAE is 1.022, and 1.075 for strain sAE-E cultures. On the other hand, strain sAE produced maximum of 50.7 mg/L of HA, while strain sAE-E produced nearly 5 times lower HA, which is only 10.3 mg/L. Since HA production increased approximately 2.5 fold over the non-aerated condition **(Prasad *et al*., 2010)**, HA production also further improved by controlled DO from 20 % to 30 % and to 50 %.

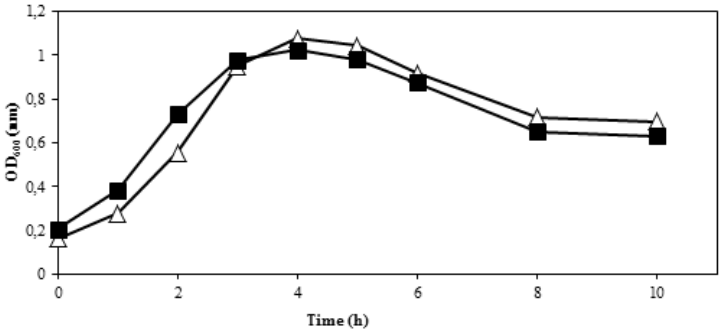

Figure 4 Growth curves of strains sAE and sAE-E in batch cultivation using 2 L of stirred tank bioreactor. (■)- strain sAE; (Δ)- strain sAE-E

The operation conditions of the process greatly affect the metabolic behavior of the production strain. The genetic manipulation themselves are not enough to make a strain achieve maximum theoretical yield. HA synthesis is a demanding process, requiring large amounts of energy. Four molecules ATP are needed for a single molecule of disaccharide repeating unit. Aerobic fermentation is therefore favorable to HA biosynthesis. A common challenge in metabolic engineering is to ensure adequate supply of precursors for target molecules. This is particularly challenging since HA synthesis tightly couples with the cell growth. Consequently, cell growth inevitably competes with the HA synthesis for the same precursor molecules. The ability to decouple these two processes will not only increase the efficiency of the processes but also allow synthesis process to be controlled independent of cell growth such that any conditions limiting cell growth may not affect the synthesis **(Mao *et al*., 2009).**

Table 3 shows the cell and HA production with different DO levels, while the impeller speed control remained the same along with the standard fermentation conditions mentioned above. Under aerobic conditions, the final cell growth are not affected by various DO levels, indicating that cell growth is unaffected by presence of oxygen, regardless the DO levels. However, HA synthesis is affected by different DO levels. The highest HA synthesis attained when the DO is controlled at 30 % which is 127 mg/L. This result can be supported by **Huang *et al*. (2006)** found that DO played an important role as a stimulant in HA synthesis and there existed a critical DO level of 5 % during HA fermentation, meaning the capacity of HA synthesis was affected below this level. Furthermore, the yield coefficient $Y_{p/x}$ was investigated, and the results are summarized in **Table 3**. There were no distinct differences in the values of $Y_{p/x}$ under 30 % and 50 %, indicating that the efficiency of the HA synthesis was unaffected when DO level is higher than 30 %. However, if compared to DO at 20 %, the $Y_{p/x}$ is about two fold lower than DO above 30 %. The highest concentration of the inclusion body protein and the volumetric titres of *Eg95* were attained at 30% DO level **(Manderson *et al.*, 2006). Chisti and Moo Young (1996)** stated that maintaining a high dissolved oxygen level in high cell density cultures requires intense agitation and aeration that can damage fragile recombinant cells.

From our results, it shown that HA biosynthesis in bioreactor under controlled pH conditions results in better glucose uptake and enhances the productivity of culture in comparison to shake-flask cultures. It is possible that the development of acid tolerance response **(Sriraman and Jayaraman, 2006)** in the shake-flask cultures due to acidification of the broth limits the energy and precursor availability for HA biosynthesis.

Molecular weight is one of key characteristic of HA product. **Chen *et al.* (2009)** have shown that in *S. zooepidemicus*, the overexpression of single genes involved in the UDP-glucuronic acid biosynthesis pathway decreased the HA molecular weight, whereas the overexpression of single genes involved in the UDP-N-acetylglucosamine biosynthesis pathway increased the HA molecular weight. In this study, the HA molecular weight was found to be sensitive to the DO level, and the results are shown in **Table 3**. At DO is 20 %, the HA molecular weight was only 54166 Da, yet it reached 70056 Da at a DO level of 30 %, and then decreased to 55041 Da at 50 %. This phenomenon was caused by the balance between HA synthesis **(Armstrong and Johns, 1997)** and oxygen-mediated degradation with various DO levels. As such, the HA molecular weight increased owing to the effect of the DO on the HA synthesis, and then decreased because of oxygen radical degradation. Thus, the HA molecular weight at 50 % DO was less than that at 30 % DO owing to oxygen radical degradation.

Cleary and Larkin, (1979) previously proposed a defense mechanism where HA capsules protect cells from oxygen in Group A, and the protective mechanism is activated in the presence of oxygen. In other words, an appropriate DO level could stimulate the synthesis of HA, and when DO level was higher than the critical level of 30 %, the stimulation effect was not so significant. Therefore, developing an efficient DO control approach was very important to improve HA productivity. However, there are no reports regarding the influence of DO control approach on the microbial HA production by recombinant *E. coli*.

Table 3 Comparison of kinetic parameters values HA biosynthesis at different controlled DO levels in a 2 L stirred tank bioreactor using strain sAE

Kinetic parameter values	20%	30%	50%
t(h)	12	8	8
OD_{600} (nm)	0.815	0.794	0.758
P_m (g/L)	0.057	0.127	0.118
S_0-S_i (g/L)	8.152	8.999	9.099
$Y_{OD/s}$ (OD/g)	0.100	0.088	0.083
$Y_{p/s}$ (g/g)	0.007	0.014	0.013
$Y_{p/OD}$ (g/OD)	0.070	0.160	0.155
P_r (g/L/h)	0.005	0.016	0.015
MW (Da)	54166	70056	55041

Fed-batch cultivation

Since HA biosynthesis of recombinant *E. coli* in our research is in simple batch culture in flask or bioreactor, it is reasonable that they are not very high. Therefore, we proceed to fed-batch cultivation by using sAE strain. In order to investigate the potential of the recombinant strain for industrial application and to probe possible limitation for HA synthesis in recombinant strains, a fed-batch fermentation process was developed. Fermentation processes of *E. coli* are commonly operated in fed-batch mode in order to prevent the accumulation of toxic substrates or products **(Lee *et al*., 1999),** thus allowing the achievement of higher product concentrations. The bacteria *E. coli* is usually grown under this kind of operation due to the well-known negative effect of acetate, which is produced when the substrate, glucose, is presented above certain concentrations **(van de Walle and Shiloach, 1998).**

In the constant feeding rate fed-batch cultivation, 10 g/L of glucose was fed into the bioreactor at a constant feeding rate of 2 ml/ min. The cell reached OD_{600} at 1.923, however the HA biosynthesis in the constant feeding rate fed-batch cultivation is only 40 mg/L, 31 % lower than batch cultivation. The comparison of batch and constant feeding rate fed-batch on HA production was provided in **Table 4**. Batch culture evidently showed the highest HA productivity on glucose, and fed-batch culture had a higher cell yield ($Y_{OD/s}$) on glucose. The average specific HA synthesis rate in fed-batch culture was 0.004 (g HA/OD/h) compared with 0.016 (g HA/OD/h) in batch culture. Therefore, it can be deduced that fed-

batch culture was more favorable for cell growth compared to batch culture, which was a more favorable way for the synthesis of HA. Fed-batch culture was chosen because of the desire to probe limitation under more practically useful conditions which lead to high titers in HA **(Mao *et al*., 2009).** The concentration of acetic acid was increased to 1.623 g/L compared with 1.407 g/L in batch culture. The average specific HA synthesis rate in fed-batch culture was 0.021(g HA/OD/h) compared with 0.16 (g HA/OD/h) in batch culture. The synthesis of acetic acid was coupled to cell growth, and thus the fed-batch culture evidenced a higher acetic acid yield as compared with that in the batch culture. In comparison of HA molecular weight in fed-batch cultivation (60881 Da) is 10 % lower than batch cultivation (70556 Da). In the fed-batch cultivation, the increased accumulation of acetic acid inhibited HA biosynthesis more severely, thus resulting in a reduced production and productivity of HA.

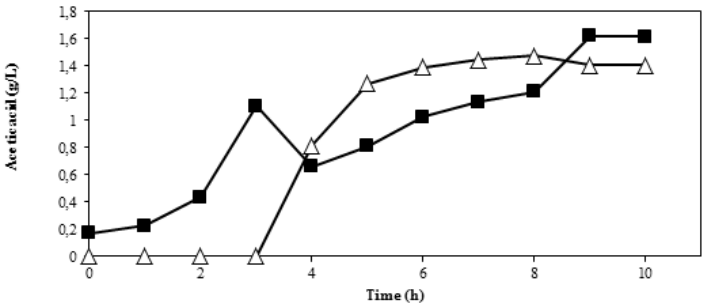

Figure 4 Acetic acid production at different mode of cultivation. (■)- fed-batch cultivation (Δ) - batch cultivation

The high cell growth in fed-batch cultivation resulted from the low inhibition of glucose in the cells. However it appeared that low glucose concentrations were not favorable for the synthesis of HA. It was previously reported that there was competition between HA synthesis and cell growth for the common precursors; UDP-glucuronic acid and UDP-N-Acetyl Glucosamine. It should be noted that cell wall is necessary for cell growth, while HA as a cell capsule is not a necessary component for cell growth. Carbon source was utilized preferably for cell growth and thus HA synthesis is inhibited, especially at low substrate concentration in fed-batch culture **(Chong *et al*., 2005).** Therefore, a lower HA productivity in fed-batch culture compared to batch culture was observed in the present study. The competition between cell growth and HA synthesis was also observed by other researchers **(Chong *et al*., 2005)**. Hence, the lower HA productivity in fed-batch culture resulted from the competition between cell growth and HA synthesis, not the decreased HA synthesis ability of *S. zooepidemicus*.

Other metabolites in the HA biosynthetic pathways such as glucose-6-phosphate and fructose-6-phosohate are consumed in the pentose phosphate pathway and glycolysis, respectively. Thus, there is stiff competition for the metabolites between HA production and cellular growth, and there is a close correlation between energy metabolism and precursor supply. As discussed above, fed-batch culture was favorable for cell growth, and cells in fed-batch culture can reach a concentration as high as that in batch culture but in a shorter time.

Table 4 Comparison of kinetic parameter values of batch and constant feeding rate fed-batch cultivation on HA biosynthesis by sAE strain

	Batch Fermentation	Fed-batch Fermentation
t(h)	8	9
OD_{600} (nm)	0.794	1.923
P_m (g/L)	0.127	0.040
AA_m (g/L)	1.407	1.623
$Y_{OD/s}$ (OD/g)	0.088	0.134
$Y_{p/OD}$ (g/OD)	0.160	0.021
P_r (g/L/h)	0.016	0.004
MW (Da)	70056	60881

CONCLUSION

In this study, the *hasA* and *hasE* genes expressed together in a plasmid in recombinant *E. coli* strains were successfully produced HA using glucose as a carbon source under aerobic condition. Production of 127 mg/L HA was observed in a batch fermentation process with the highest HA molecular weight (70056 Da) using strain sAE in 2 L stirred tank bioreactor with controlled DO at 30%. The fed-batch culture with constant feeding of glucose was favorable for cell growth and the highest OD_{600} achieved at 1.923. However, the HA biosynthesis and molecular weight in fed-batch culture were lower than that observed in the batch culture. Further optimization of fermentation process and control strategies should lead to an even higher yield.

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GLUCOSE CONSUMPTION AND LACTIC ACID FORMATION IN MILLET SOURDOUGH FERMENTED WITH DIFFERENT STRAINS OF LACTIC ACID BACTERIA

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ABSTRACT

The consumption of glucose and production of lactic acid by selected autochtonous strains of facultatively heterofermentative *Lactobacillus coryniformis*, *Pediococcus pentosaceus* and *Pediococcus acidilactici* species, in millet sourdoughs was studied. Glucose consumption and lactic acid concentration were analyzed after 24 h, 48 h and 96 h of sourdough incubation. They varied significantly depending on the strain. The highest production of lactic acid in all fermentation steps was found in sourdough fermented with *Pediococcus acidilactici* strain, whereas the lowest amount of this metabolite was found in sourdoughs fermented with *Pediococcus pentosaceus* ZFP5. Lactic acid concentration decreased in the successive fermentation steps and after 96 h of sourdoughs fermentation its level was of about 44 % lower than after 24 h of incubation. Glucose was completely consumed in all samples after 96 h of fermentation.

Keywords: lactic acid, millet, sourdough, *Lactobacillus coryniformis*, *Pediococcus pentosaceus, Pediococcus acidilactici*

INTRODUCTION

The increasing demand for baked products with a high nutritional value or health benefits is raising the need for the use of alternative (non-wheat) cereals like rice, maize, millet, and sorghum in the baking industry. The alternative cereals have received extensive scientific and technological attention due to better health-promoting composition, especially regarding minor components present in grains (dietary fiber, resistant starch, minerals, vitamins, phenolic compounds) (**Coda *et al.*, 2014**). The greatest advantage of the alternative cereals is a lack of gluten, the causative agent for celiac disease (**Moroni *et al.*, 2009**).
Among the alternative cereals, millet is one of the oldest cultivated plants. In ancient times, it was prevalent in Asia, Africa and central regions of Europe. Millet is unique due to its short growing seasons and its capability of producing good yields of grain under conditions unfavorable to most other cereals. Millet is desirable in man's diet because it is easily digestible and is a rich source of various macroelements including calcium, potassium, magnesium, sodium and valuable unsaturated fatty acids. Moreover, millet contains B vitamins, especially niacin, B6 and folic acid (**Verma *et al.*, 2013**). In terms of protein content and fat, millet grains outweigh popular oat and buckwheat grains. The energy of millet grains is similar to that of rice, buckwheat, but their nutritional values are higher. Effects of millet proteins are comparable to the biological value of proteins of wheat, maize, and beans. Owing to its valuable nutritional values, millet has received a renewed interest among consumers of the European countries. It can be used in several ways, e.g., it may be ground into flour and made into a host of different non-yeast breads. Millet grains and flour are commonly used for making fermented products. Traditional foods prepared with natural fermentation of millet are: gruels, porridges, soups, and fermented beverages. Millet flour can also be used for sourdough preparation. However, the use of the millet-based sourdough in the baking industry is not well standardized. Millet, like the other alternative cereals, is characterized by a low baking quality and final sensory quality (**Gallagher *et al.*, 2004**). These disadvantages limit the use of such flour in the bread making process. One of the ways to overcome these problems is the improvement of fermentation technology, with the main challenge being the design of well-defined starter cultures with functional features for sourdough preparation. It has been reported that lactic acid bacteria fermentation of alternative flours has a relevant influence on sensory as well as baking qualities, providing final products with desirable properties (**Coda *et al.*, 2010**). The huge benefit of the consumption of the alternative flour-based bakery products on health together with increasing demand for healthier products affords researchers the opportunity to find new microbial biodiversity for food processing. Therefore, an increasing number of studies has been recently observed that address the lactic acid bacteria (LAB) microbiota of alternative cereals. On the basis of these studies, it has been found that the selected autochthonous lactic acid bacteria are one of the best candidates to ferment the sourdough. Sourdough fermentation with such species leads to an increased value of *in vitro* protein digestibility and to improvement of the nutritional and sensory potential of non-wheat grains (**Coda *et al.*, 2011; Sterr *et al.*, 2009**). It was also observed that the type of cereals plays an important role in starters selection (**Moroni *et al.*, 2010**). During the selection of the proper starter cultures, consideration should also been given to the sensory characteristics of final products. Therefore, studies on the biochemical pathways of lactic acid bacteria leading to the formation of flavor compounds and their precursors are necessary.
The objective of our study was to evaluate the behavior of the selected facultatively heterofermentative lactic acid bacteria strains i.e.: two different strains of *Lactobacillus coryniformis*, three strains of *Pediococcus pentosaceus* and one strain of *Pediococcus acidilactici* in millet sourdoughs. In particularly, the production of lactic acid by the selected strains was investigated.

MATERIAL AND METHODS

Microorganisms

Six lactic acid bacteria strains (LAB), previously isolated form homemade millet sourdoughs, were used in this study: *Lactobacillus coryniformis* ZFP1 and ZFP4; *Pediococcus pentosaceus* ZFP2, ZFP3 *and* ZFP5*; Pediococcus acidilactici* ZFP6. These strains were identified by 16S rDNA sequencing. LAB were grown anaerobically in MRS medium at 30 °C.

Sourdough preparation and fermentation

Millet flour (Bio Babalscy, Poland) was used for fermentation of sourdoughs. Sourdoughs were prepared by mixing sterile tap water and whole meal flour of millet in a 1:1 (w/w) ratio. The dough yield was 200. The fermentations were carried out at 25 °C for 24h. Sourdoughs were inoculated with 0.5% of starter culture (10^{10} cfu/g dough). The fermentations were continued with 3 back-sloppings (once every 24 h) at 25 °C using 10% of the ripe sourdough as the inoculum. After 24h of initial fermentation and at the 1st and 3rd refreshment step, samples were taken from the ripe sourdough and analysed. An uninoculated (control) sourdough was prepared under the same conditions. Data were obtained from two independent sourdough fermentations.

Lactic acid and glucose analysis in sourdough

All chemicals used in the study were from Sigma-Aldrich. The chemicals were all of analytical grade.
Prior to analyses, dough extracts were treated as described previously (**Robert *et al*., 2006**) with some modifications. Sourdough was homogenized with redistilled water (1:7). Then, the solution was stirred with 2.5 mL of Carrez I solution and 2.5 mL of Carrez II solution and was centrifuged for 3 min at 150 rpm. The supernatant was filtered through a 0.45 µm filter (Membrane Solutions, USA) prior to analysis.
Glucose and lactic acid were quantified by HPLC apparatus (Gilson, Inc. Meddleton, USA) with an Aminex 87HP column (300 mmx 7.8 mm, Bio-Rad, Mississauge, Canada) at a temperature of 20 °C and a flow rate of 0.6 mL min^{-1} with H_2SO_4 (pH = 3.4) as the eluent. The quantification was based on a refractive index detector and performed with external standards in duplicate. The results were expressed as mean values. Errors are represented as standard deviations.

Statistical analysis

Data were compared by Tukey's test. Statistical significance ($p<0.05$) was determined with STATISTICA (Statsoft) software.

RESULTS AND DISCUSSION

The selected autochthonous LAB strains of *Lactobacillus coryniformis*, *Pediococcus pentosaceus* and *Pediococcus acidilactici* applied in this study are facultatively heterofermentative microorganisms that degrade mainly hexoses via the Embden-Meyerhof-Parnas (EMP) pathway and the lactic acid is the major end product (Gänzle et al. 2007). *Pediococcus pentosaceus* and *Pediococcus acidilactici* are important LABs involved as starter cultures in meat, vegetable and dairy fermentation and causing characteristic flavor changes, improving hygienic quality and extending the shelf life of several products (Irmler et al. 2013). *Lactobacillus coryniformis* has been recently reported to display a variety of potential probiotic properties, to be characterized by a strong antifungal activity and potential to be used as a biopreservative in feed systems (Magnusson, Schnurer, 2001; Sekwati-Monang et al. 2012).
Glucose consumption and lactic acid formation in millet sourdoughs are depicted in Table 1.

Table 1 Glucose utilization and lactic acid formation after 24 h, 48h (1st refreshment) and 96 h (3rd refreshment) of millet sourdough fermentation. Sourdoughs were inoculated with approximately 10^{10} cfu/g of each of the six strains, incubated at 25 °C and back-slopped every 24 h with 10% inoculum. Results are shown as means ± standard deviation of duplicate independent experiments analyzed in duplicate.

Strains	Incubation time [h]	Glucose utilization [mmol kg^{-1}]	Lactic acid formation [mmol kg^{-1}]
L. coryniformis ZFP1	24h	8,5 ± 0,44 a B	130,4 ± 5,41 a F
	48h	46,4 ± 1,09 b C	116,0 ± 8,13 b D
	96h	n.d.	65,8 ± 1,33 a AB
L. coryniformis ZFP4	24h	18,4 ± 0,61 b D	99,9 ± 3,49 b D
	48h	n.d. A	87,3 ± 2,18 a C
	96h	n.d.	65,5 ± 1,08 a AB
P. pentosaceus ZFP2	24h	n.d. A	89,0 ± 2,50 a C
	48h	63,2 ± 0,20 D	106,0 ± 2,72 a AB
	96h	n.d.	54,2 ± 3,37 a BD
P. pentosaceus ZFP3	24h	47,7 ± 2,90 a G	115,4 ± 5,01 b E
	48h	n.d. A	105,4 ± 1,08 a AB
	96h	n.d.	70,3 ± 3,81 b AC
P. pentosaceus ZFP5	24h	26,3 ± 0,75 b E	76,0 ± 3,12 c B
	48h	n.d. A	67,2 ± 1,54 b E
	96h	n.d.	41,9 ±1,46 a D
P. acidilactici ZFP6	24h	14,7 ± 1,29 C	180,9 ± 5,71 G
	48h	16,1 ± 0,21 B	113,5 ± 4,15 BD
	96h	n.d.	77,8 ± 3,81 AC
control (uninoculated dough)	24h	35,9 ± 0,80 F	52,8 ± 1,94 A
	48h	n.d. A	96,7 ± 2,98 AC
	96h	n.d.	79,7 ± 7,73 C

n.d.-not detect
*Means between strains of the same LAB species followed by the different small letters and means between the different LAB species followed by the different capital letters are significantly different at $p < 0,05$ (Tukey test)

In the studied millet sourdoughs, glucose was the dominant utilized carbohydrate. The consumption of glucose, as a major carbon source, was observed in sorghum sourdough (Sekwati-Monang et al. 2012). After the first 24 h of fermentation, the level of glucose varied depending on the LAB strains used. Moreover, statistically significant differences were observed between strains of the same LAB species (Table 1). In case of *P. pentosaceus* strains ZFP3 and ZFP5, the level of glucose was 47.7 and 26.3 mmol kg^{-1}, respectively, whereas no glucose was detected in sourdough fermented with *P. pentosaceus* ZFP2. Significantly lower amounts of glucose were found in sourdoughs inoculated with *L. coryniformis* ZFP1 and ZFP4 (8.5 and 18.4 mmol kg^{-1}), and *P. acidilactici* (14.7 mmol kg^{-1}). Glucose level in the spontaneously fermented millet dough was relatively high (35.9 mmol kg^{-1}), compared to the inoculated samples.
The amount of synthesized lactic acid, after 24 h of fermentation, ranged between 76.0 and 180.9 mmol kg^{-1}, depending on the strain used. The highest amount was detected in the sourdough fermented with *P. acidilactici* ZFP6 (180.9 mmol kg^{-1}), whereas the lowest was found in sourdough with *P. pentosaceus* ZFP5 (76.0 mmol kg^{-1}).
After 48 h of incubation (1st refreshment), a successive decrease of lactic acid was observed in almost all sourdoughs, except the sample inoculated with *P. pentosaceus* ZFP2 and the non-inoculated one. The differences in the amount of produced lactic acid between the strains of the same LAB species as well as between different LAB species diminished and in few cases became statistically less significant. No significant differences were observed between the sourdoughs fermented with *P. pentosaceus* ZFP2 and *P. pentosaceus* ZFP3. However, significantly lower amount of lactic acid was synthesized in sourdough fermented with *P. pentosaceus* ZFP5. Compared to the other millet sourdoughs, *L. coryniformis* ZFP1 and *P. acidilactici* ZFP6 exhibited the highest level of lactic acid production.
Considering glucose consumption after 48h of incubation, its level varied between the samples and in case of the control and sourdough fermented with *L. coryniformis* ZFP4, *P. pentosaceus* ZFP3, and *P. pentosaceus* ZFP5 was not detected. In other samples, its level was higher compared to the samples obtained after 24h of incubation.
During 96 h of sourdough fermentation glucose was completely consumed in all the studied samples. The level of lactic acid ranged from 41.9 mmol kg^{-1} to 79.7 mmol kg^{-1}, and was of about 44% lower than after the first 24h of fermentation. No statistically significant differences were observed between the sample inoculated with strains of the *L. coryniformis* species as well as between the *P. pentosaceus* ZFP2 and *P. pentosaceus* ZFP5.
Carbohydrate metabolism of LAB and formation of lactic acid during sourdough fermentation is a very complex process. The nature of sourdough microbiota as well as the type of flour used are one of the main factors determining the metabolite kinetics of sourdough fermentation. The metabolic activity of various

LAB species and their interaction with other sourdough microorganisms are flour specific. The unique chemical composition of wheat, rye and non-wheat flours affect the observed varying behavior of the species participating in the fermentation process. Moreover, the observed differences in the carbohydrate metabolism are not only between the strains of various LAB species but also between the strains of the same LAB species. In our study, statistically significant differences in glucose and lactic acid level between the applied selected LAB starters were detected predominantly after 24h of fermentation. During the next fermentation steps, the differences diminished and finally at the end of fermentation were almost comparable. The observed behavior of the applied autochthonous starters results probably from their interaction and competitive activities with other millet sourdough microorganisms.

CONCLUSION

The present study has shown varied behavior of selected autochthonous LAB starters during millet sourdough fermentation. Among the used starters, *P. acidilactici* ZFP6 exhibited the highest level of lactic acid production in all fermentation steps, compared to the other inoculated sourdoughs. Whereas, the lowest amount of this metabolite was found in sourdoughs fermented with *Pediococcus pentosaceus* ZFP5.
The obtained results will be a useful tool to better understanding of the carbohydrate metabolism of LAB in the millet sourdough and to the further studies on design of proper LAB starter cultures for millet sourdough preparation.

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PERFORMANCE AND MOLECULAR IDENTIFICATION OF BACTERIA ISOLATED FROM THE GUT OF BROILER BIRDS AFTER ANTIBIOTIC ADMINISTRATION AND ENZYME SUPPLEMENTATION

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ABSTRACT

This study evaluated the effect of feed additives (antibiotic or enzyme) on performance and bacteria population in the ileum of one day – old broiler chicks (ANAAC 2000) randomly distributed to three treatments having eight replicates and ten birds per replicate. Completely randomized design was used and experiment lasted for 35 days.
Maize–soybean meal diet without antibiotic administration or enzyme supplementation served as control and was the same diet for all *treatments*. Antibiotic (Dicoxin plus ®) was administered to birds fed diet 2 and enzyme (Roxazyme G 2G ®) was supplemented to diet 3.Bacterial specific primers for rRNA gene sequences were used to amplify bacterial genes from samples and sequenced. Bacteria were identified from the gene sequences using Basic Local Alignment Search Tool (BLAST) against the National Centre for Biotechnology Information (NCBI).
Enzyme supplementation significantly ($P<0.01$) improved final Live body weight and weight gain compared to control or administration of antibiotic. The FCR was significantly enhanced ($P<0.01$) by enzyme and antibiotic supplementation. *Lactobacillus acidophilus (L. acidophilus)*, *Escherichia coli* (*E. coli*) and *Clostridia* were identified in digesta sampled. Partial rRNA sequences identical to *Clostridia* were the lowest (1) in control and enzyme treatment. A value of 4 was recorded in antibiotic treatment. *Lactobacillus acidophilus* was numerically high in control (8) and enzyme treatment (5) compared to antibiotic administration (1). Partial rRNA sequences identical to *Escherichia coli* sequences was however high (48) in birds administered antibiotic compared to control (8) and enzyme treatment (9). Results indicated greater improvement in weight gain, FCR and *Lactobacillus* in broilers fed enzyme supplemented diet. Feed additives may affect the biodiversity of gut bacteria in poultry birds.

Keywords: antibiotics, broilers, enzyme supplementation, molecular identification, performance

INTRODUCTION

Supplementing poultry feed with specific enzymes improves the nutritional value of feed ingredients by increasing the efficiency of digestion and nutrient uptake. These enzymes help to increase the availability of nutrients particularly starch, protein, amino acids and minerals such as phosphorus and calcium from feed ingredients. Variability in the nutrient content of maize has been demonstrated to be as great as that observed for wheat and barley (**Leeson *et al*., 1993; Collins *et al.,* 1998**). In practice, the average nutrient content of cereals is greater in the presence of enzymes than its absence. As a result, the addition of an enzyme allows feed formulation nutrient matrix values to be elevated. The response to enzyme addition is mediated through improvements in nutrients extraction in the small intestine by the host through accelerated digestion, reduced microbial activity as a result of substrate limitation in the ileum and active feeding of specific bacterial species. Essentially, the activity of the enzyme on viscous polymers and cell wall carbohydrates produce sugars and oligomers which are utilized preferentially by certain ileal and caecal bacterial species. The bacterial species flourish at the expense of other possible detrimental species as far as optimal growth or health of animal is concerned (**Apajalathi and Bedford, 1999**).
The importance of understanding the dynamics of intestinal microbial ecology has been recognized for a long time (**Savage, 1977**). Since the ban of in–feed antibiotic growth promoters, the concept of gut health, interaction between gut microbes and nutrient bioavailability in relation to bird performance has become important. Digestive disorders have increased in parallel to this withdrawal (**Van Immerse *et al*., 2004**). This is often a source of underperformance due to health problems such as *necrotic enteritis* or *coccidiosis* (**Williams, 2005**). Currently, there is increasing focus on alternatives to sustain good gut flora and gut health. Potential alternatives that may be suitable include enzymes, probiotics, prebiotics, essential oils, botanicals and organic acids. Several of these products have been widely tested and the evaluation will continue in the future. These alternatives exert beneficial gut health effects on the host (**Ravindran, 2012**), but the effects of their administration on animal performance have been reported to be variable (**Partanen and Mroz, 1999; Dibner and Buttlin, 2002; Patterson and Burkholder, 2003; Ricke, 2003; Dibner and Richards, 2005; Gianneanas, 2008; Yang *et al*., 2009**).
Maize and soybean meal are used in feeding broiler chickens worldwide. It is almost free of viscous non–starch polysaccharides (NSP) but this does not necessarily exempt enzyme use in diets containing both ingredients. Maize and soybean meal contain appreciable amounts of NSP. According to **Bach Knudsen (1997),** it contains approximately 0.9% soluble NSP and 6% insoluble NSP. According to earlier reports (**Noy and Sklan, 1995; Thrope and Beal, 2001),** corn starch digestibility rarely exceeds 85% in broilers between 4 and 21 days of age indicating opportunities for improvement. Insoluble fibre shortens retention time of digesta (**Hetland *et al.,* 2004**) and may lead to nutrient digestibility. Unlike soluble fibre, their effect on population and quantity of microflora is relatively important (**Hetland *et al.,* 2004),** although the increase of digesta passage time probably reduces settle time for fermentative microbes (anaerobic organism) especially in the small intestine.
The role of gut health in performance of poultry birds has resulted in the use of several feed additives. Gradually the use of in-feed antibiotics is no longer a favourable alternative to alleviate digestive disorders and poor performance associated with its withdrawal (**Van Immerse *et al.,* 2004).** Although these suitable alternatives (enzymes, probiotics, prebiotics, symbiotics, *etc*.) have been reported to elicit varied results in terms of growth performance, however, how they affect the biodiversity of gut bacteria may be an issue of consideration.
Due to lack of knowledge of appropriate culturing conditions, a large number of bacteria remain unidentified using basic culturing and biochemical methods. Furthermore, culturing and biochemical techniques have resulted in the misclassification of some of these bacteria **(Tellez *et al.,* 2006).** Given the profound impact of gut bacteria on performance, identification of bacteria assemblages in the gut at random will be of high biological and economic importance (**Apajalathi and Bedford, 1999; Apajalathi *et al.*, 2001, 2004; Ezenwa *et al.*, 2012).**

In the light of this, this study was designed to assess the performance of broilers fed with or without antibiotic and enzyme supplemented diet and their effects on *Lactobacillus* counts in the ileum. Considering the limitation of standard culturing methods, this study also aimed at identifying bacteria biodiversity amplified from the gut of broiler birds through molecular techniques.

MATERIAL AND METHODS

This experiment was carried out at the poultry unit of Niger Delta University Teaching and Research farm, Niger Delta University, Wilberforce Island, Nigeria.

Composition of experimental Diet

The composition of the experimental diets is presented in Table 1 below;

Table 1 Gross composition of experimental diets (g/kgDM unless otherwise stated)

Ingredients	M / SBM Diet 1(control)	M / SBM + antibiotic Diet 2	M / SBM + enzyme Diet 3
Maize	550	550	550
Soybean meal	330	330	330
Fish meal	40	40	40
Cassava starch	42	42	42
*Constant ingredients	38	38	38
Total (1000gm)	1000	1000	1000
M.E. (Kcal/kgDM)	3024	3024	3024
C.P	214.94	214.94	214.94

Legend: mineral vitamin premix (2.5g), DL Methionine (1.5g), bone meal (21g), oyster shell (10g) salt (3g). M.E.: metabolisable energy, C.P.: crude protein, M: maize, SBM: soybean meal.

Three experimental diets were formulated. All the diets were maize–soybean meal based (M/SMB), which contained 550g/kg of maize. The control diet was not supplemented with enzyme or the birds given antibiotics. An antibiotic (Dicoxin plus ®) was administered to birds fed with diet 2 at an inclusion rate of 100g/160 litres via drinking water. Diet 3 was supplemented with enzyme (Roxazyme G 2G ® - DSM Nutritional Products Ltd, Switzerland). The gross composition of the experimental diets is as indicated in Table 1.

All the birds were fed with the same type of diet except the treatment administered (i.e. antibiotic administration and enzyme supplementation). Energy and crude protein concentration was similar for all treatments and was adequate for the birds under each treatment. A hundred gram of each experimental diet was collected and set aside for proximate analysis. Proximate analysis of experimental diets was carried out according to **AOAC (1990)**.The nutrient composition of the experimental diets is presented in Table 2.

Table 2 Nutrient contentof experimental diets (in g/kg DM unless otherwise stated)

Nutrient	M/SBM	M/SBM + antibiotic	M/SBM + enzyme
Dry matter (g)	739.5	733.5	651.5
Ash	154.2	148.1	170.2
Crude protein	238	229	256
Ether extract	58.1	62.7	49.1
Crude fibre	64.9	73.69	70.6

M/SBM: maize / soybean meal

Source of enzyme

The enzyme used in the current study is a non starch polysaccharide (NSP) degrading enzyme and was supplemented at an inclusion rate of 200g/t of complete feed. It is an odorless granulates which is soluble in water. It contains an enzyme complex derived from *Trichoderma longibrachiatum* with an effective pH range of 3.5 – 5.5and a temperature range of 30 – 55°C. The specifications of the enzyme are:

- Endo-1,4-glucanaseactivity: min 8,000 unit per gram (E.C.3.2.1.4.)
- Endo-1,3 (4)-glucanase activity: min 18,000 unit per gram (E.C.3.2.1.6.)
- Endo-1,4-xylanase activity: min 26,000 units per gram (E.C. 3.2.1.8.)

Animal experiment

A total of two hundred and forty (240) ANAAC 2000 one day-old broiler chicks were purchased brooded for seven days and randomly distributed to the three dietary treatments having eight replicates of ten birds per replicate. The experiment was arranged as a completely randomized design. Feed and water were supplied *ad libitum*. Since the focus of the experiment was not on the use of antibiotics, antibiotics and anticoccidiostats were not administered to the birds fed with the control diet and the enzyme supplemented diet. This was to determine the effect of exclusive antibiotic administration and enzyme supplementation on beneficial gut bacteria (*Lactobacillus*) as well as the overall performance of birds fed with a standard maize/soybean meal based diet. Feed intake and weight gain were determined on a weekly basis, while feed conversion ratio (FCR) was calculated. The antibiotic administration to birds fed with the second diet was done for 6 days from day 21 to 26. This was stopped on day 27 and discontinued till the end of the experiment. The duration of the experiment was 35 days.

Digesta collection

On day 35, two birds per replicate were slaughtered and digesta was collected to determine *Lactobacillus* counts in the ileum. The ileum was defined as 2cm posterior to merkels diverticulum and 2cm anterior to the ileal – caecal – colonic junction. After a rapid removal of this section of the gut, digesta was collected into sterile sample containers on ice. The digesta collected was taken to the laboratory for microbial analysis.

Microbial analysis

Lactobacillus was enumerated on bacteria specific agar (de man Rogossa and Sharp agar – MRS agar) after prior serial dilution of 1gm wet weight of collected digesta. The diluents were plated out in duplicate per replicate and incubated for 48 hours after which *Lactobacillus* colonies were counted. *Lactobacilli* counts were log transformed before carrying out statistical analysis.

Bacteria identification

Four birds were randomly slaughtered from each treatment and digesta was collected from the ileum into sterile sample containers and stored on ice packs. The digesta was stored at -20°C prior to molecular analysis. Metagenomic DNA was extracted and purified using ZR Fungal/Bacterial DNA MiniPrep™50 Preps. Model D6005 (Zymo Research, California, USA) according to the Manufacturer's protocol. The DNA samples were thereafter sent to Inqaba Biotechnology Pretoria South Africa for Polymerase Chain reaction (PCR) and sequencing according to **Weisburg *et al.* (1991).** The 16S rRNA partial gene sequence were targeted and amplified through PCR using primers (27-F and 1492-R) with sequences being 5'-AGA GTT TGA TYM TGG CTC AG-3' and 5'-TAC CTT GTT AYG ACT T-3' respectively **(Martin and Collen,1998)**.The resulting DNA sequences were subjected to Basic Local Alignment Search Tool (BLAST) analysis on the National Centre for Biotechnology Information (NCBI) platform on the web and bacterial isolates were identified based on the resultant top hits(**Altschul *et al.*, 1990)**.

Data collection and analysis

Data collected include weight gain, feed intake and calculated feed conversion ratio (FCR) in addition to data collected on log CFU *Lactobacillus* counts were subjected to general linear model analysis using SPSS package (SPSS Inc, Chicago, IL. 2008) version 17 and significant means separated using Duncans

Multiple Range test (**Steele and Torrie, 1995**).

RESULTS AND DISCUSSION

Analyzed crude protein and ash concentration was higher in enzyme supplemented diet as shown in Table 2. This could be attributed to hydrolysis of NSP and release of minerals and proteins attached to high molecular weight carbohydrates. The crude protein concentration was the least (229g/kgDM) in diet 2 to which antibiotic were administered to the birds fed this diet. A value of 256g/kgDM was recorded in the enzyme supplemented diet which was the highest value recorded across the treatments. The value recorded for the control diet was 238g/kgDM. A similar trend was observed for the ash concentration across the treatments respectively. The best crude protein concentration recorded in the enzyme supplemented diet compared to the control and antibiotic administered diet is in line with previous work reported by **Ohimain and Ofongo (2013)** an increased crude protein concentration in an enzyme supplemented maize/soybean meal based diet containing 200g of wheat offal. Although the maize does not contain viscous NSP, enzyme supplementation can improve birds' performance by increasing digestibility of nutrients found in maize kernel (**Meng and Slominski, 2005**). A probable mode of action of Roxazyme G2G with regards to the enzyme supplemented diet could be via the hydrolysis of certain types of carbohydrate-protein complexes (glycoproteins, proteoglycans) in which the protein component is resistant to proteolysis because of its substitution with bulky carbohydrate groups **(Shibuya and Iwasaki, 1985; Meng and Slominski, 2005**).In addition, the release of such proteins from high molecular weight carbohydrate-protein complexes could enhance protein availability for digestion and absorption.

Table 3 Performance and *Lactobacillus* counts (*log CFU*) in broilers fed with feed additive supplemented diets

Performance indices g/bird except FCR	M/SBM	M/SBM+ antibiotic	M/SBM+ enzyme	SEM	*P value*
Initial live weight	138.33	148.75	143.75	-	-
Final live weight	1909.70^{a}	2249.50^{b}	2464.30^{c}	64.76	0.000***
Weight gain	1771.30^{a}	2103.29^{b}	2320.80^{c}	65.44	0.000***
Feed intake	3811.84	3700.96	4072.23	130.23	0.143^{ns}
FCR	2.19^{b}	1.77^{a}	1.78^{a}	0.072	0.001***
Gut section (ileum)					
Lactobacillus	7.58^{bc}	7.33^{a}	7.76^{c}	0.06	0.034**

abc: means along the same row with different superscripts are significantly different (*P<0.05*)
M/SBM: maize-soybean meal; FCR: feed conversion ratio; SEM: standard error of mean

Results on performance variables (Table 3) showed significantly higher final live body weight and weight gain (*P<0.01*) in broilers fed with enzyme–supplemented diet compared to antibiotic administration or no supplementation at all. From the results, antibiotic administration and enzyme supplementation significantly (*P<0.01*) improved weight gain and FCR than feeding the maize/soy bean meal diet without either treatment.

Enzyme supplementation significantly (*P<0.01*) improved the performance of broilers while increasing the *Lactobacillus* counts in relation to antibiotic administration. Antibiotic administration also significantly (*P<0.01)* enhanced weight gain compared to the control diet but values recorded was significantly (*P<0.01)* lower than enzyme supplementation. The least value of weight gain (1771.30g) was recorded in birds fed the control diet while values of 2103.29g and 2320.80g was recorded for birds given antibiotic and birds fed enzyme supplemented diet respectively. According to **Ofongo *et al.* (2011)** and **Ikoro(2010)** improved weight gain was reported in broilers fed enzyme supplemented maize – soybean meal based diet. The current findings further substantiate earlier report by **Cowieson (2005)** that demonstrated an improved FCR from 0.78% to 10.5% and body weight gain from 0.5% to 10.9% in enzyme supplemented maize based diet over the control. Apparently, low viscosity diets which are considered to be energy dense can have their nutrient availability improved. In another report (**Chesson, 2001**), maize kernel was stated to contain 111g/kg of total NSP of which 230g/kg is arabinose and 300g/kg is xylose. That report further warrants the use of exogenous enzymes to increase the digestibility of nutrients found in maize kernel (**Meng and Slominski, 2005**). The enzyme used in the current study had enzyme activities (over 26,000 units per gram of xylanase activity) which may have been adequate in hydrolyzing the NSP present in maize kernel. Although feed intake was not significantly (*P>0.05*) different across the treatments, however, antibiotic use and enzyme supplementation significantly (*P<0.01*) enhanced FCR compared to the control diet. Feed intake value was least in birds administered antibiotic, with a value of 3811.84g recorded in the control and 4072.23g in birds fed enzyme supplemented diets. According to **Tahir *et al.* (2005),** cellulase and hemicellulase and their combination increased body weight gain without having any effect on feed intake in broilers fed corn/soy bean meal based diet. Although maize-soy bean based diets do not induce high intestinal viscosity as other cereals, it has been shown that these diets could benefit from carbohydrase- supplementation when fed to broilers (**Cowieson, 2010**). Results from various studies however, have been to some extent inconsistent (**Zanella *et al*., 1999; Centeno *et al.*, 2006; Singh *et al.*, 2012**). The results of this study are similar to that reported by **Tahir *et al.* (2005)** but this was not the case with that observed by **Cowieson and Ravindran (2008).** The authors observed increase in both body weight gain and feed intake in response to enzyme supplementation with xylanase, amylase and protease. In this regard the enzyme cocktail may be a probable factor since amylase and protease were not part of the enzyme component in the enzyme used in this study. **Tahir *et al*. (2008)** reported a 9% gain in body weight of broilers fed diets with enzyme combination of cellulose, hemicelluloses and pectinase. The report of **Olukosi *et al.* (2007)** observed no effect on body weight gain in broilers fed enzyme supplemented corn/soy bean based diet.

The adequacy of nutrients or nutritional values of all diets used in this study were adequate since all birds in the 3 treatments were given a similar diet. Expectedly any response observed may be attributed to treatment effect and not variability in diet composition. It was suggested by **Cowieson (2010**) that there are many interacting factors involved in dictating the measured response to an exogenous enzyme of which the most influential is the nutritional value of the diet to which the enzyme is added. Furthermore, broilers fed diets that are essentially adequate in all nutrients often still respond to exogenous enzyme supplementation (**Bao *et al*., 2013**). The authors suggested that enzyme benefits may be due to changes in less tangible metrics such as appetite control, digestive physiology, immunology or microbiology i.e. net effects. Maize is a highly digestible feed ingredient due to its low NSP concentration thereby bringing to the fore the inherent digestibility of nutrients in the diet prior to enzyme addition. This digestibility has been demonstrated to be a good indicator of the magnitude of the enzyme response (**Cowieson and Bedford, 2009; Cowieson, 2010**). This was obvious in birds fed diets 2 and 3. In the case of diet 2, antibiotic administration minimized competition for nutrients between host and gut microorganisms. Enzyme supplementation on the other hand must have improved nutrient digestibility via reduction in cell wall integrity, generation of fermentable disaccharides, low-molecular weight polysaccharides and oligosaccharides, improving protein solubility, decreasing endogenous losses and overcoming anti-nutritional factors (**Cowieson and Ravindran, 2008**).

The mechanism of antibiotic use is apparently to control intestinal microflora favoring beneficial bacteria while suppressing detrimental or pathogenic bacteria that provoke inflammation of the gut mucosa. As a result, antibiotics are used routinely in poultry to prevent and treat diseases associated with gut microflora. With the ban on their use in most developed countries, the poultry industry is faced with the challenge of controlling pathogenic microbes. Modulation of the gut microflora can be done either through diet or enzyme supplementation (**Ohimain and Ofongo, 2013**), which could stimulate and encourage the growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacteria.* Due to the fact that microbial colonization of the gut takes place post hatch, a large number of microorganisms can become established in the GIT shortly after hatching. Intestinal microflora is mainly responsible for degrading the plentiful amounts of mucus produced by the goblet cells in the intestine (**Falk *et al.*, 1998**). A less or not inflamed gut with little or no bacteria competing for nutrients and less digestive disorders mean better nutrient digestion and absorption with a resultant enhanced weight gain. This might have been the probable reason for observed weight gain in birds administered antibiotics.

Lactobacillus counts (Table 3) was significantly influenced (*P<0.05*) by antibiotic administration and enzyme supplementation. It was significantly (*P<0.05*) higher in birds fed with enzyme supplemented diet than antibiotic administration even though the antibiotic was not targeted at *Lactobacillus.* This report is congruent with previous findings (**Ohimain and Ofongo, 2013**). *Lactobacillus* counts were numerically higher in enzyme supplemented diet than the control diet but this apparent difference was not significant (*P>0.05*) with the control. Gut microflora has significant effect on host nutrition, health and growth performance (**Barrow, 1992**) by interacting with nutrient utilization and the development of gut ecosystem of the host. This interaction is very complex and depending on the composition and activity of gut microflora, it can have either positive or negative effect on the health and growth of birds. For example, when pathogens attach to the intestinal mucosa, gut integrity and function are severely affected (**Droleskey*et al.*, 1994**) and immune system is threatened (**Neish, 2002**). According to **Klasing *et al*. (1987**), chicks grown in pathogen free environment grow 15% faster than those under conventional conditions where they are exposed to bacteria and viruses. The high number of *Lactobacillus* in the ileum indicated an acidic environment which could prevent pathogenic bacteria from colonizing the GIT and enhance performance with less competition for nutrients

with the bird. It is generally agreed that gut microflora is a nutritional "burden" in fast growing broiler chickens (**Dibner and Richard 2004**; **Lan*et al.*, 2005**). The focus of alternative strategies is to prevent proliferation of pathogenic bacteria and modulation of indigenous bacteria so that the health, immune status and performance of broilers can be improved **(Ravindran, 2006)** as demonstrated in the present study.

Previous study by **Tierlynck *et al.* (2009)** showed evidence and markers of gut damage, apoptosis, increased mounting of immune defense and microbial invasion of intestinal tissues in broilers fed wheat–ryediet compared to corn. This does not rule out the microbial response in the gut of broilers fed maize based diet in the presence and absence of NSP enzyme or antibiotics. Carbohydrase supplementation has been shown to reverse the negative effects of NSP mediated by gut microorganisms **(Hogberg and Lindberg, 2004; Kiarie *et al.*, 2007).** This response is mediated by increasing the proportion of lactic and organic acids, reducing ammonia production (**Kiarie *et al.*, 2007**) and increasing volatile fatty acid (VFA) concentration as reported by **Huberner *et al.* (2002**). According to the author, increased VFA concentration is an indicative of hydrolysis fragmentation of NSP and this supports growth of beneficial bacteria. Increased proportion of lactic acid promotes gut health by suppressing growth of presumptive pathogens (**Pluske *et al.*, 2001; Ohimain and Ofongo, 2013**). **Hillman *et al.* (1995**) observed that certain strains of *Lactobacillus* inhibit the growth of *coliforms* such as *E. coli*. The obviously improved *Lactobacillus c*ount in birds fed enzyme supplemented diet above antibiotic administration is indicative of the added benefit of enzyme addition to a maize-soybean meal diet. Also, in the absence of enzyme, *Lactobacillus*count was significantly better than antibiotic administration. **He *et al.* (2010)** reported that xylose (possible product of exogenous and endogenous carbohydrase activity) is important in preferentially enhancing the growth of beneficial *Bifidobacteria.* According to **Torok *et al.* (2007)** and **Courtin *et al.* (2008),** exogenous enzymes mediated changes that influenced gut microbial populations. The results of this study sheds light on the impact of antibiotic use and enzyme supplementation on *Lactobacillus* population in the gut and overall growth response of broilers to their application. **Bedford and Cowieson (2012)** suggested that intestinal microbial population size and composition clearly plays a very large role in determining the extent of digestion accomplished by the host and by extension, growth rate and efficiency.

Table 4 Relative number specific Bacteria identified in ileum content of broiler gut as affected by feed additives

Treatments	Bacteria genera		
	Lactobacillus acidophilus	*Escherichia coli*	*Clostridia*
Control	8	8	1
Antibiotic	1	48	4
Enzyme	5	9	1

The number of bacteria of economic importance according to their specific species identified using molecular techniques is presented in Table 4. Results obtained for molecular identification of gut bacteria (Table 4) showed that the relative number of *E. coli* identified was numerically high in the antibiotic treatment compared to the control and enzyme treatment. This was also the case with *Clostridia* which was least in both the control and enzyme treatment. The relative number of *Lactobacillus acidophilus* was the least in the antibiotic treatment but was numerically higher in treatment 1 and 3 respectively. Results previously reported using bacteria specific culturing methods showed that feed additives significantly affected *lactobacillus* population in the ileum (**Abule *et al.*, 2014)**. Although the antibiotic used in this study had anti-coccidia properties, molecular techniques of identification revealed the presence of *Clostridia* population in the gut of birds administered an antibiotic which was numerically higher than the control and enzyme treatment. Although *E. coli* is part of the normal flora in the lower section of the intestine of warm blooded animals, the relative number identified via molecular means in the current study was also numerically higher than that obtained in the control and enzyme treatment. Observed disparity in the relative number of *Lactobacillus acidophilus* recorded further buttresses the report of **Abule *et al.* (2014)**, which stated that antibiotics enhance performance of broilers but may not necessarily increase *Lactobacillus* counts in the gut.

According to **Apajalathi *et al.* (2004)**, recent molecular studies targeting bacterial DNA in poultry gut have yielded more detailed insight into the composition of the diverse microbial community. Furthermore, this composition may be further diversified depending on usage or non-usage of feed additives. Results of the current study also shed light on the principle of competitive exclusion in gut microflora population based on nutrient availability in the gut. Modulation of the gut microflora either through diet and enzyme supplementation (**Ohimain and Ofongo, 2013**) may stimulate or encourage the growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacteria.* In spite of this, the bacteria biodiversity in the gut may also be influenced by feed additives depending on nutrient availability and population of specific bacteria present in the gut.

The interaction between gut microflora, nutrient utilization and development of the gut ecosystem of the bird is complex and can affect performance depending the composition and activity of the gut microflora. This interaction can have either positive or negative effect on health and growth of birds. For example, when pathogens attach to the intestinal mucosa, gut integrity and function can be severely affected (**Droleskey *et al.*, 1994**) and the immune system threatened (**Neish, 2002**). As previously stated **Klasing *et al.* (1987**), chicks grown in pathogen free environment grow 15% faster than those under conventional conditions where they are exposed to bacteria and viruses. The high number of *Lactobacillus* in the ileum as earlier stated indicated an acidic environment which will not favour pathogenic bacteria and better performance with less competition for nutrients with the bird. The focus of alternative strategies to prevent proliferation of pathogenic bacteria and modulation of indigenous bacteria so that the health, immune status and performance are improved (**Ravindran, 2006**) as indicated in the present study is a very welcomed idea.

CONCLUSION

It can be concluded from the findings of the current study that antibiotics enhance performance of broilers but may not necessarily increase *Lactobacillus* counts in the gut. Enzyme supplemented maize–soybean meal based diet improves weight gain as well as *Lactobacillus* counts in the gut. In-feed antibiotics may not favour the proliferation of beneficial bacteria when administered to prevent digestive disorder in broiler birds. It is of benefit to the farmer to supplement maize – soybean meal based diet with enzyme rather than antibiotic where such alternative is available. Molecular identification of gut bacteria under different additive supplementation may shed more light on the role of gut bacteria on performance, physiology of the gut and overall health of broiler birds.

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IN VITRO AND IN SILICO ANTIBACTERIAL ACTIVITY OF PRANGOS *FERULACEA* (L.) Lindl AND *PRANGOS ULOPTERA* DC, AND THEIR MUTAGENICITY IN THE AMES TEST

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ABSTRACT

The present study was conducted to study antibacterial activity of different extracts of *Prangos ferulacea* and *Prangos uloptera*. The antibacterial activity was measured by disc diffusion and micro-broth dilution methods at different concentrations (250, 500, 750, 1000, 1500, 2000, 2500 and 3000µg/ml).The studied bacteria were *Streptococcus pyogenes, Staphylococcus aureus, Bacillus subtilis, Serratia marcescens, Escherichia coli, Salmonella enterica*.The in sillico antibacterial activity of pinens and coumarins was performed by Autodock 4 software. The molecular docking between phytochemicals and six target proteins (DNAgyrase subunit B, pinicilin binding protein, D-alanin D-alanin syntase, dihydrofolate reductase, and dihydropteroate synthetase and isoleucyl-tRNA sinthetase) has been investigated. The mutagenicity of these extracts at different concentrations (500, 1000, 1500, 2000, 2500, 3000µg/ml) were also investigated on *Salmonella typhimurium* strain TA98. The results confirmed that all tested extracts have modest to weak antibacterial activity against studied bacteria without any mutagenicity effect.The root and seed extracts of both species respectively had highest and lowest antibacterial effects.The antibacterial activity of pinens of these plants was significantly more than coumarins. DNA gyrase subunit B and penicilin binding proteins (PBP) were the main targets of tested coumarins. DNA gyrase subunit B was also the main target of studied pinens.Our study found that *P.ferulacea* and *P.uloptera* displayed a great potential of antibacterial activity.

Keywords: Antibacterial activity, Molecular docking, Mutagenicity, *Prangos ferulacea, Prangos uloptera*

INTRODUCTION

In recent decades the activity of conventional antibiotics against pathogenic bacteria has decreased due to the expansion of bacterial resistance (**Adwan *et al.*., 2010**).However,for some decades there was an increasing interest to screen plants constituents which have antimicrobial activities (**Al-Akeel *et al.*., 2014**).These compounds act on the cell wall, protein synthesis, and DNA replication during the bacterial division cycle (**Alves *et al.*., 2014**).Some plants constituents have been reported to possess potent mutagenic effect (**Eren and Özata, 2012; Akintonwa *et al.*, 2009; Dos-Santos *et al.*., 2011**).So it is necessary to investigate the mutagenic effect of natural compounds. In silico methods have also been used to identify drug targets. Molecular docking is one of the best bioinformatics tools for drug design that used extensively by scientists.Molecular docking could determine the binding affinity of a ligand for a target protein.This technique has been used extensively for discovery of plant phytochemicals with antimicrobial activity (**Kroemer, 2007; Zoete *et al.*., 2009**).Therefore we became eager to assess different constituents of prangos genus with respect to their antibacterial activity.The genus *Prangos* that known Djashir in Iran belongs to the Smyrneae tribe from the Apiaceae family.These plants widely used in folk medicine to treat external bleeding, anti-worm, healing scars,digestive disorder and leukoplakia (**Rahimi *et al.*., 2014; Razavi *et al.*., 2011**).*Prangos ferulacea* and *Prangos uloptera* are two species of this genus that are distributed from east Europe to central and eastern Asia (**Razavi, 2012; Abolghasemi and Piryaei, 2012**).So far, many studies have been done on the medicinal properties of these plants including anti-bacterial and anti viral, anti fungal, ani cancer and anti diabetic activity (**Kafash-Farkhad *et al.*., 2013**).However the mechanism of antibacterial activity of Jashir constituents has been not investigated yet. Some coumarins and pinens have been reported to be present in the root extracts of *P.ferulacea* and *P.uloptera* (**Baser *et al.*., 1996; Sefidkon and Najafpour, 2001**).In the present study for first time antibacterial and mutagenesis effects of methanolic extracts from different parts of *P.ferulacea* and *P.uloptera* and probable antibacterial mechanism(s) of dominant reported phytochemicals consist of coumarins and pinens from these plants have been studied.

MATERIAL AND METHOD

Plant materials

Plant materials were collected from Kurdistan province in Iran, during the period between May and June 2014. The identities of the plants were confirmed by botanist at the Herbarium of the University of Isfahan, Iran.

Preparation of plant extracts

The samples were separated into flower, leaf, stem, seed and root parts.The plant parts were dried in shadow and powdered. The methanol extracts were prepared by macerating 100 g of powdered plant material in 300 ml of methanol, for 72 h and filtered using Whatman filter paper. The extraction was done thrice at room temperature.The collected solvents were concentrated by rotary vacuum evaporator (Stero glass, Italy) at 45°C and then dried using a freeze dryer (Zirbus, Germany). All extracts were dissolved in dimethyl sulphoxide (DMSO) and diluted to give concentrations of 250, 500, 1000, 1500, 2000, 2500, 3000 µg/ml (**Behbahani and Sadeghi-aliabadi, 2013**)

Bacterial strains

Bacterial strains were purchased from the Iranian Biological Resources Center and Bio Reliance Corporation (Rockville,MD,USA).The strains used in this study were *Streptococcus pyogenes* (ATCC:1447), *Staphylococcus aureus* (ATCC:25923), *Bacillus subtilis* (ATCC:6633), *Serratia marcescens* (ATCC:1111), *Escherichia coli* (ATCC:25922), *Salmonella enterica* (ATCC:14028) and *Salmonella typhimurium* TA98. The bacterial strains were grown on Nutrient Broth medium at 37˚C for 8 h.

In vitro antibacterial activity

Inhibition of bacterial growth by the plant extracts was evaluated by disk diffusion assay (**Dunkelberg, 1981**).The sterile Whatman filter papers No.1 were prepared and soaked separately in each of the extracts for 5 min.The filter papers

placed on the plate. After 24 h of incubation at 37 °C, the zone of inhibition around the each disc was measured. The MIC values were also determined by micro-dilution method **(Eloff, 1998).** Briefly, the plant extracts were serially diluted and added to a 96-well plate. 100 µl of an appropriate medium (*Mueller-Hinton Broth*) and 20 µl of the inoculums (containing about 6×10^4 colony) were dispensed into each well of a 96-well plate. After 24 hour incubation period at 30°C, plates were read at 620 nm. MIC value is defined as the lowest concentration which inhibits the growth or fewer than 3 discrete colonies were detected.On the other hand MBC value was defined as the lowest concentration of the plant extracts to kill the microorganisms.Plates were read in triplicate, and the average MIC value was recorded.

Docking study

Ten active compounds in *P.ferulacea* root has been selected from previous reports including: α-pinen, β–pinen, Gosferol, Terpinolen, Myrcene, P-cymene, δ-3-caren, Pesoralen, Osethole and Isoimperatorin.All these compounds were subjected to molecular docking studies for inhibition of antibiotic target proteins. In the present study 6 target proteins consist of DNAgyrase subunit B (DGSB with PDB entry 3TTZ), Pinicilin binding protein (PBP1a with PDB entry 3UDI), D-alaninD-alanin ligase (DdL with PDB entry 2ZDQ), dihydrofolate reductase (DHFR with PDB entry 3SRW),dihydropteroate synthetase (DHPS with PDB entry 2VEG) and isoleucyl-tRNA sinthetase (IARS with PDB entry 1JZQ) have been chosen for docking study.Also performed molecular docking between six standard antibiotics(Ciprofloxasin,Benzylpenicillin, Sulfadiazine, Trimethoprim, D-cycloserine, Mupirocin) with mention targets as the positive control.The 3D structure of mentioned compounds and standard antibiotics was obtained from Pub Chem (http://pubchem.ncbi.nlm.nih.gov) database as SDF format.The 3D structure of mentioned target proteins was also obtained from protein data bank as PDB format. Molecular docking was performed using Autodock4 (version 4.2) with the Lamarckian genetic algorithm.Docking parameters which selected for AutoDock4 runs were as follows:100 docking runs, population size of 200, random starting position and conformation, translation step ranges of 2A, mutation rate of 0.02, cross-over rate of 0.8, local search rate of 0.06 and 2.5 million energy evaluations. Docked conformations were clustered by a tolerance of 2 Å root mean square deviations (RMSD).

Mutagenicity assay

The Mutagenic effect of flower, leaf, stem, seed and roots of *P.ferulacea* and *P.uloptera* on S.typhimurium TA98 were studied by plate incorporation assay procedure at different concentrations (250,500,1000,1500,2000,2500 and 3000 µg/ml). Briefly, 100 µl of an overnight grown culture (10^7 CFU/ml) added in sterile screw capped tubes. Then, 2 ml of top agar and 100 µl of each extract were added to the tubes and the tubes were vortexed. Then the solution was poured onto a minimal glucose agar plate. Then plates were incubated at 37 °C and the number of His+ revertant colonies was counted after 48h.The positive and negative controls in this assay were sodium azide and 1-3% DMSO respectively. The minimal glucose agar plate contained 1.5% agar, 2.0% glucose, and 2.0% Vogel-Bonner medium.The top agar was consisted of 0.6% agar and 0.5%NaCl. The triplicate plating was used for each extract. The mutagenic effect of different compounds was estimated using the twofold rule according to the following formula. The substance is considered mutagen if the QM higher than 2**(Nosrati and Behbahani, 2015).**

QM =Number of His+ revertant colonies from tested extracts/colonies from negative control

RESULTS

In vitro antibacterial assay

The results of disc diffusion method showed that all plant extracts have antibacterial activities against the mentioned bacteria (Table 1-3). The antibacterial activities of different parts of studied plants were dose dependent. The results demonstrated that the antibacterial activity of *P.ferulacea* extracts is significantly more than *P.uloptera* extracts.The root extracts of both species were found to have the higher antibacterial effect compared to the flower, leaf, stem and seed extracts.The MIC and MBC values of all extracts were further estimated and are shown in Table 4. Among these bacterial strains, E.coli was susceptible to all plant extracts with MIC value ranges of ≤250 to 3000 µg/ml. Based on the results, the root extracts of both species showed highest antibacterial activity with MIC values ranges of ≤250 to 1000 µg/ml against tested strains. The antibacterial activity of the extracts was followed by flower, leaf, stem, and seed in both species.Based on these results and in comparison to MIC and MBC values of standard antibiotics ,MIC value at ≤250µg/ml was judged to show high antibacterial activity, while 500-1000 µg/ml were considered to show moderate and ≥3000 µg/ml weak antibacterial activity.

Table 1 Antibacterial *activity* of flower, leaf, stem seed and root extracts of *P.ferulacea* and *P.uloptera* against gram positive bacteria (Inhibition zone are mean±SD)organism

	Concentration(µg/ml)	Inhibition zone(mm)									
		P.ferulacea					*P.uloptera*				
		flower	leaf	stem	root	seed	flower	leaf	stem	root	seed
S. aureus	250	-	-	-	6±0.22	-	-	-	-	7±0.21	-
	500	7±0.11	-	-	7±0.20	-	7±0.13	-	-	8±0.28	-
	750	8±0.10	-	-	7±0.28	-	7±0.18	-	-	8±0.34	-
	1000	8±0.23	6±0.22	-	9±0.40	-	9±0.52	8±0.24	7±0.13	10±0.30	-
	1500	8±0.22	7±0.24	-	9±0.36	-	9±0.21	8±0.22	9±0.12	10±0.37	-
	2000	10±0.40	9±0.28	6±0.25	11±0.26	7±0.16	11±0.13	9±0.26	9±0.16	11±0.12	6±0.22
	2500	12±0.45	11±0.10	7±0.11	13±0.20	7±0.24	13±0.24	10±0.11	10±0.32	12±0.17	7±0.12
	3000	15±0.18	13±0.30	8±0.18	15±0.18	9±0.27	14±0.28	12±0.25	10±0.20	15±0.50	8±0.18
B. subtilis	250	6±0.17	-	-	7±0.24	-	6±0.22	6±0.28	-	6±0.11	-
	500	6±o.24	-	-	7±0.26	-	6±0.32	8±0.25	-	7±0.37	-
	750	8±0.16	6±0.16	-	8±0.32	-	9±0.36	8±0.52	-	8±0.31	-
	1000	8±0.44	8±0.18	-	10±0.30	-	9±0.35	9±0.23	-	10±0.32	-
	1500	9±0.11	8±0.32	8±0.33	11±0.21	6±0.25	10±0.30	11±0.12	6±0.22	11±0.37	7±0.32
	2000	10±0.23	9±0.52	8±0.28	13±0.25	7±0.32	12±0.38	12±0.26	7±0.43	13±0.30	7±0.23
	2500	12±0.26	11±0.26	9±0.18	14±0.15	8±0.34	13±0.42	15±0.20	8±0.27	13±0.39	9±0.42
	3000	14±0.36	13±0.32	12±0.13	16±0.12	11±0.37	14±0.44	15±0.28	9±0.41	16±0.62	9±0.40
S. pyogenes	250	-	-	-	6±0.27	-	-	6±0.26	-	6±0.32	-
	500	-	-	-	7±0.30	-	7±0.30	8±0.34	-	8±0.30	-
	750	7±0.11	-	-	7±0.27	-	7±0.28	10±0.30	6±0.50	8±0.12	-
	1000	8±0.15	-	-	9±0.24	-	9±0.11	10±0.25	7±0.27	9±0.25	-
	1500	8±0.25	7±0.13	-	10±0.20	-	10±0.19	11±0.22	7±0.32	11±0.42	-
	2000	9±0.34	8±0.37	-	10±0.24	-	10±0.36	12±0.25	9±0.20	11±0.36	-
	2500	11±0.30	10±0.40	7±0.24	11±0.36	7±0.11	12±0.23	12±0.23	10±0.17	12±0.27	-
	3000	12±0.27	10±0.12	7±0.26	13±0.12	8±0.22	12±0.26	13±0.26	11±0.20	14±0.20	7±0.21

Table 2 Antibacterial effects of flower, leaf, stem seed and root extracts of *P.ferulacea* and *P.uloptera* against gram negative bacteria (Inhibition zone are mean±SD)

organism	concentration	Inhibition zone(mm)									
		P.ferulacea					*P.uloptera*				
		flower	leaf	stem	root	seed	flower	leaf	stem	root	seed
S.marcescens	250	-	-	-	-	-	-	-	-	-	-
	500	6±0.21	-	-	7±0.28	-	6±0.21	6±0.44	-	7±0.25	-
	750	7±0.23	6±0.21	-	8±0.26	-	8±0.25	6±0.36	-	9±0.33	-
	1000	8±0.32	8±0.16	6±0.38	8±0.14	-	8±0.31	7±0.40	-	9±0.40	-
	1500	8±0.11	9±0.31	7±0.34	9±0.12	-	9±0.43	8±0.23	-	11±0.28	-
	2000	10±0.25	9±0.16	8±0.37	10±0.18	6±013	10±0.26	8±0.62	6±0.34	13±0.47	-
	2500	12±0.22	10±0.26	10±0.52	13±0.15	8±0.14	11±0.36	9±0.32	8±0.60	14±0.55	7±0.47
	3000	13±0.27	12±0.33	13	14±0.12	9±042	13±0.19	10±0.12	9±0.52	16±0.20	8±0.35
E.coli	250	6±0.11	-	-	7±0.14	-	-	-	-	7±0.37	-
	500	7±0.27	6±0.15	-	8±0.16	-	-	7±0.27	-	9±0.40	-
	750	8±0.25	7±0.32	-	8±0.25	-	7±0.15	8±0.33	-	10±0.36	-
	1000	9±0.33	8±0.41	6±0.21	9±0.28	-	8±0.32	9±0.40	-	11±0.13	-
	1500	10±0.42	8±0.20	7±0.26	11±0.32	-	10±0.52	10±0.37	-	12±0.27	-
	2000	10±0.28	9±0.26	8±0.32	12±0.30	-	11±0.42	10±0.44	-	12±0.37	6±0.60
	2500	11±0.20	10±0.40	9±0.23	13±0.26	-	12±0.17	11±0.50	7±0.21	14±0.26	7±0.16
	3000	13±0.21	12±0.13	10±0.25	14±0.29	-	13±0.47	12±0.47	8±023	16±0.30	8±0.46
S.enterica	250	-	-	-	6±0.33	-	7±0.32	-	-	7±0.34	-
	500	7±0.28	-	-	7±0.40	-	8±0.33	-	-	9±0.50	-
	750	8±0.42	6±0.15	-	9±0.52	-	9±0.27	6±0.35	-	9±0.25	-
	1000	9±0.17	7±0.16	6±0.41	9±0.34	-	10±0.26	7±0.10	6±0.36	11±0.32	-
	1500	11±0.20	8±0.23	7±0.35	10±0.33	6±0.37	11±0.54	8±0.13	7±0.18	13±0.41	-
	2000	11±0.61	10±0.24	9±0.22	11±0.22	8±0.39	12±0.47	8±0.19	9±0.25	14±0.55	-
	2500	12±0.42	11±0.28	11±0.26	13±0.17	9±017	13±0.19	9±0.32	9±0.10	14±0.39	7±0.18
	3000	13±0.21	13±0.30	11±0.42	15±0.33	10±0.28	14±0.38	11±0.24	9±0.26	16±0.10	8±0.43

Table 3 Inhibition zone (mm) and MIC (µg/ml) values of 5 standard antibiotics (Ampicillin, Penicillin, Gentamicin, Streptomycin, and Ciprofloxacin). Ciprofloxacin and Penicillin were effective antibiotics, IZ in this table is the abbreviation of Inhibition zone

Organism	Standard antibiotic									
	Ampicillin		Penicillin		Gentamicin		Streptomycin		Ciprofloxacin	
	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC
S.aureus	19±0.21	250	23±0.63	125	19±0.18	500	20±0.42	125	22±0.36	125
B.subtilis	22±0.33	125	21±0.47	125	17±0.40	500	21±0.58	125	24±0.28	125
S.pyogenes	20±0.40	125	20±0.36	125	21±0.38	125	18±0.36	250	21±0.25	125
S.marcescens	18±0.51	250	18±0.28	125	19±0.15	500	15±0.40	750	20±0.33	125
E.coli	16±0.27	500	18±0.30	250	17±0.10	500	18±0.68	500	19±0.25	250
S.enterica	17±0.16	250	17±0.45	250	15±0.50	750	15±0.13	500	17±0.14	250

Table 4 MIC (µg/ml) and MBC (µg/ml) values of flower, leaf, stem seed and root extracts of *P.ferulacea* and *P.uloptera*

Microorganism												organ	plant
S.aureus		*S.pyogenes*		*B. subtilis*		*E.coli*		*S.enterica*		*S.marcescens*			
MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC		
<1000	2000	<1500	2500	<750	1500	250	500	<750	1500	750	1500	leaf	*P.ferulacea*
<500	1000	750	2000	250	500	<500	1500	<500	1500	500	1000	flower	
2000	>3000	<2500	>3000	1500	>3000	1000	2000	<1000	2500	<1000	2500	stem	
250	500	250	500	<250	500	250	500	250	500	500	1000	root	
2000	>3000	<2500	>3000	1500	3000	>3000	>3000	1500	>3000	<2000	>3000	Seed	
<1000	2000	<250	500	250	500	250	500	750	1500	500	1000	leaf	*P.uloptera*
500	1500	500	1000	250	500	500	1500	<250	750	<500	1000	flower	
1000	1500	750	2000	1500	>3000	<2500	>3000	1000	2000	<2000	>3000	stem	
<250	750	250	500	250	500	250	500	250	500	<500	1000	root	
<2000	>3000	<2500	>3000	<1500	2500	<2000	>3000	<2500	>3000	2500	>3000	Seed	

Mutagenicity assay

Mutagenicity effect of methanol extracts of *P.ferulacea* and *P.uloptera* are shown in Table 4. These extracts were tested under comparable conditions at different concentrations (250, 500, 1000,1500,2000,2500, 3000 µg ml^{-1}).The results demonstrated that both plant extracts didn't have any mutagenicity effect and the QM values of all tested extracts were calculated less than 2. However, the numbers of TA98 His+ revertant colonies in plates treated with *P.ferulacea* extracts were higher than *P.uloptera* extracts.The plates treated with leaf extract of *P.ferulacea* was also showed highest revertant colonies with QM values of 1.68.

Table 5 QM values of extracts obtained from separate parts of *P.ferulacea* and *P.uloptera* tested on TA98: The highest numbers of TA98 His+ revertant colonies was observed in plates treated with3000 µg/ml of the leaf of the *P.ferulacea*

Mean Qm of different extracts ±SD						Part of plant	Plant species
3000	2500	2000	1500	1000	500		
1.44±0.09	1.39±0.1	1.35±0.04	1.29±0.04	1.25±0.03	1.23±0.06	**leaf**	
1.48±0.1	1.43±0.06	1.39±0.04	1.37±0.2	1.36±0.06	1.34±0.08	**flower**	
1.49±0.04	1.46±0.07	1.40±0.03	1.37±0.03	1.34±0.02	1.32±0.03	**root**	***P. uloptera***
1.40±0.02	1.38±0.02	1.31±0.04	1.29±0.04	1.27±0.09	1.25±0.07	**stem**	
1.38±0.03	1.35±0.06	1.28±0.09	1.26±0.1	1.23±0.06	1.20±0.09	**seed**	
1.68±0.1	1.66 ±0.1	1.46 ±0.07	1.40 ±0.09	1.26 ±0.1	1.13 ±0.1	**leaf**	
1.48±0.1	1.43 ±0.09	1.33 ±0.09	1.26 ±0.08	1.16 ±0.09	1.10 ±0.08	**flower**	
1.50±0.08	1.46 ±0.08	1.33 ±0.06	1.26 ±0.09	1.26 ±0.08	1.20 ±0.09	**root**	***P. ferulacea***
1.58±0.05	1.53 ±0.1	1.46 ±0.07	1.40 ±0.05	1.33 ±0.09	1.26 ±0.11	**stem**	
1.64±0.1	1.60 ±0.09	1.46 ±0.07	1.40 ±0.08	1.23 ±0.09	1.10 ±0.13	**seed**	

INSILICO ANALYSIS OF ANTIMICROBIAL ACTIVITY

The results of docking study of six antibiotic target proteins with the mentioned phytochemicals and standard antibiotics have been showed in Table 5. The results demonstrated that all studied compounds had appropriate interaction to antibiotic targets.Results also showed that all compounds had RMSD less than 2.Analysis of docking results showed that both DNA gyrase subunit B and penicillin binding protein (PBP) were the main targets for tested coumarins. DNA gyrase subunit B was also the main target for studied pinens. The other targets for pinens and coumarins were respectively D-alanin D-alanin ligase(Ddl), dihydrofolate reductase (DHFR), dihydropteroate synthetase (DHPR) and isoleucyl-tRNA sinthetase (IARS).The patterns of amino acids interaction of DNAgyrase subunit B with the mentioned compounds have been shown in figur1.Molecular docking revealed that 11 amino acids of DNA gyrase subunit B interact with the mentioned compounds.Among these amino acids, Asn54, Glu58, Asp81, Gly85, Ile86 and Thr173 appeared to interact with all compounds.Based on the results, α-pinen had highest efficacy for tested target proteins with ΔGb and ki values in (-7.5 to -11.2 kcal/mol) and (21.2-32.4µM) spectra respectively. Among coumarins osethole had the highest efficacy, it was followed by pesoralen, isoimpratorin, Terpinolen, Gosferol, δ-3-caren, P-cymene and Myrcene respectively.

Table 6 In silico analysis of the antibacterial activity of dominant compounds in the root of *P.ferulacea* with the highest antibacterial activity, in compared to standard antibiotics.The main target for pinens and coumarins studied were DNA gyrase subunit B and penicilin binding proteins (RMSD (Å)-Ki (µM)-ΔGb (kcal/mol)).

Compound	Target proteins																	
	DGSB			PBP1a			DHFR			DHPR			Ddl			IARS		
	RMSD	Ki	ΔGb	RMSD	Ki	ΔGb	RMSD	Ki	ΔGb	RMSD	Ki	ΔGb	RMSD	Ki	ΔGb	RMSD	Ki	ΔGb
α-pinen	0.19	21.2	-11.2	0.35	21.1	-10.3	0.40	29.5	-9.4	0.47	30.2	-8.5	0.33	31.4	-8.2	0.30	32.4	-7.5
β-pinen	0.27	34.4	-10.3	0.43	23.4	-10.1	0.34	36.3	-9.1	0.51	35.6	-8.4	0.40	37.2	-7.3	0.32	34.6	-7.2
Osethole	0.33	36.3	-9.6	0.48	37.3	-9.2	0.20	37.2	-8.7	0.28	38.9	-8.2	0.43	39.1	-7.1	0.38	41.7	-6.3
Pesoralen	0.46	41.7	-9.4	0.56	43.4	-8.6	0.76	43.2	-8.3	0.18	44.1	-7.9	0.57	44.5	-6.4	0.46	45.3	-5.4
Isoimpratorin	0.52	44.3	-8.1	0.68	46.2	-8.2	0.81	47.8	-7.6	0.43	48.2	-6.4	0.63	47.3	-6.3	0.60	48.2	-5.1
Terpinolen	0.61	48.2	-7.4	0.90	51.2	-7.1	0.93	51.8	-6.4	0.76	52.3	-6.1	0.74	54.2	-5.5	0.71	57.2	-4.8
Gosferole	0.74	51.3	-6.2	0.93	54.9	-6.3	1.05	53.9	-6.3	0.87	54.6	-5.3	0.86	56.8	-5.3	0.75	58.3	-4.3
δ-3-caren	0.80	55.7	-5.3	0.98	57.4	-5.2	1.09	57.4	-5.9	0.94	58.9	-4.4	0.90	59.2	-4.2	0.93	63.1	-3.8
P-cymene	0.91	57.3	-5.2	1.06	59.3	-5.1	1.12	59.2	-5.5	1.14	61.2	-4.2	0.95	62.9	-3.7	0.97	65.2	-3.2
Myrcene	1.06	68.9	-4.7	1.11	71.6	-4.6	1.27	71.6	-5.3	1.20	73.4	-3.7	1.21	75.6	-3.2	1.12	80.2	-31
Ciprofloxasin	0.23	16.2	-13.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-2.8
Benzylpenicillin	-	-	-	0.17	14.7	-12.5	-	-	-	-	-	-	-	-	-	-	-	-
Sulfadiazine	-	-	-	-	-	-	-	-	-	0.14	19.3	-11.8	-	-	-	-	-	-
Trimethoprim	-	-	-	-	-	-	0.18	20.5	-11.3	-	-	-	-	-	-	-	-	-
D-cycloserine	-	-	-	-	-	-	-	-	-	-	-	-	0.07	15.7	-11.2	-	-	-
Mupirocin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.15	19.2	-12.3

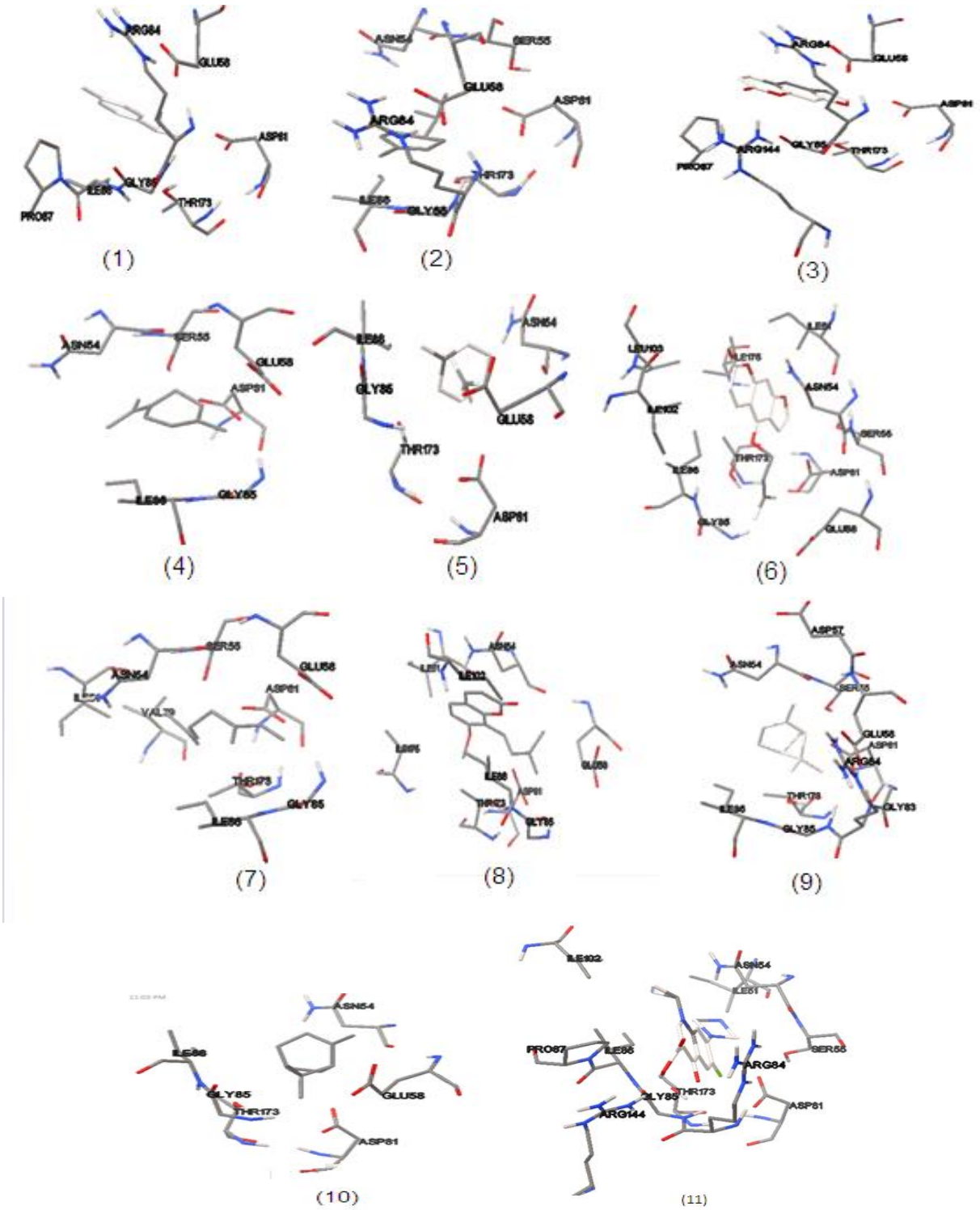

Figure 1 Molecular docking study between 10 chemical compounds and ciprofloxasin with DNAgyrase subunit B us mainly target for all compounds.1: p-cymen and DNAgyrase subunit B, 2:gosferol ,3:psoralen ,4:terpinolen , 5:δ-3-caren, 6:isoimpratorin, 7:myrecen ,8:osthole ,9:α-pinen ,10:β-pinen ,11:ciprofloxasin

DISCUSSION

The results of the present study demonstrated that methanol extracts of different parts of *P.ferulacea* and *P.uloptera* have modest to weak antibacterial activity against *S.aureus, B.subtilis, S.pyogenes, S.marcescens, E.coli and S.enterica.*The results also showed that antibacterial activity of *P.ferulacea* was significantly more than *P.uloptera*. Several researches have been done on antibacterial activity of aerial parts of *Prangos* species against Pathogenic bacteria such as: *B.cereus, B.subtilis, M.luteus and S.*aureus (**Durmaz *et al*, 2006; Massumi *et al.*, 2007**).Previous studies have also reported that α-pinens and coumarins are dominant constituents in essential oils of different parts of *P.ferulacea* and have significant antibacterial activity (**Baser *et al.*, 1996**).Some studies demonstrated that pinens and coumarins are main phytochemicals in roots of prangos species (**Sefidkon *et al.*, 1998; Sajjadi *et al.*, 2011**).In the present study, the root extracts of prangos species have the most antibacterial effect compared to leaf, stem and root extracts.It may be due to the accumulation of coumarins in root extracts of these two species in comparison with stem and leaf extract. Previous studies demonstrated that coumarins of *Prangos pabularia* and *P.uloptera* have significant anti bacterial and anti fungal activity (**Razavi *et al.*, 2008; Tada *et al.*, 2002**). Razavi *et al* **(2010)** also showed that dichloromethane (DCM) extract and different coumarins derivative from *P.uloptera* root collected from Ardebil province of Iran has high antibacterial properties against Staphylococcus aureus and Bacillus subtilis, whereas our results showed that methanolic extract from *P.uloptera* root exhibited modest antibacterial activity on mentioned strains. So the location of plant growth and extract type can affect the antibacterial properties of this plant. Although the antibacterial potential of *P.ferulacea* and

P.uloptera has been demonstrated in several studies but the antibacterial mechanisms of the active constituents of these plants have not well defined. Molecular docking is one of best bioinformatic tools for drug design and determination the mechanism of antimicrobial agents (**Kumalo *et al*, 2015**).In the present study, the docking between 10 known compounds from *P.ferulacea* and bacterial proteins has been done.The results showed that the antibacterial activity of pinens of this plant was significantly more than coumarins. DNAgyrase subunit B was the main target proteins of pinens.The α-pinen was more effective than β-pinen with lowest and highest Ki and ΔGb values respectively.Pinens (α-pinene & β-pinene) are hydrocarbon compounds that, well known chemicals having antimicrobial activity (**Dorman and Deans, 2000**). The previous study confirmed that enantiomers of α-pinene, β-pinene have antibacterial activity (**Da-Silva *et al.*, 2012**).Several results also have been presented the effectiveness of pinenes against molds and pathogen yeasts and bacteria (**Moreira *et al.*, 2007; Leite *et al.*, 2007**).DNA gyrase subunit B and penicilin binding proteins (PBP) were the main target proteins of tested coumarins.Among the tested coumarins,osethole was most effective which followed by Pesoralen,Isoimpratorin, Terpinolen,Gosferol, δ-3-caren, P-cymene, and Myrcene respectively.Coumarins are secondary metabolites that occur naturally in several plant families and possess important pharmacological properties, including inhibition of oxidative stress and use as the fragrance in food and cosmetic products (**Borges *et al.*, 2014**).The antibacterial activity of some coumarins such as osthol,*imperatorin*, isoimpinellin.arbutin,baicalin and naringin have been reported previously (**Widelski *et al.*, 2009; Ng *et al.*, 1996**).The mechanisms of antibacterial effect of these compounds have not well defined and the present study is the first investigation of the mechanism of antibacterial activity of coumarins and pinens.Despite the therapeutic advantages possessed by medicinal plants but some constituents of medicinal plants have been shown to be potentially mutagenic,toxic, teratogenic and carcinogenic (**Gadano *et al.*, 2006; Akinboro and Bakare, 2007**).Therefore, these plants should be evaluated to better understand their safety.The Ames test is commonly used with plant extracts for possible gene mutation determination (**Mortelmans *et al.*, 2000**).In this study the Ames test was carried out using methanolic extracts of flower, leaf, stem, root and seeds from *P.ferulacea* and *P.uloptera* obtained results have not shown any mutagenicity to TA98 for both studied plants. Some studies demonstrated that different pinens and coumarins have not any mutagenicity effect. Gomes-Carneiro *et al* confirmed that beta-myrcene, alpha-terpinene, (+,-) alpha-pinene have not mutagenic effect in the Ames test **(Gomes-Carneiro *et al*, 2005)**. Also, another study *revealed* that different coumarin derivatives have not any mutagenic effect on peripheral blood, liver, bone marrow and testicular cells of Swiss albino mice by the comet assay **(de Souza Marques *et al*, 2015)**. On the other hand some study confirmed the mutagenic and co-mutagenic effect of coumarins while as yet mutagenic effect induced by pinens not reported. In this regards some studies reported that Coumarin modulates the mutagenic effects of other chemicals such as aflatoxin B1 and heterocyclic amines **(Sanyal *et al*, 1997; Goeger *et al*, 1999; Kim *et al*, 2005).**

CONCLUSION

The results of this study confirmed that the methanolic extracts of different parts of *P.ferulacea* and *P.uloptera* especially roots extracts present potential antibacterial activity without any mutagenic effect.In silico analysis of antibacterial effect also showed that pinens and coumarins of mentioned plants play key roles in appearance antibacterial activity with inhibition of DNA gyrase subunit B and penicilin binding proteins respectively.Based on these results *P.ferulacea* and *P.uloptera* are good candidates for discovering bioactive compounds in the form of the antibacterial agents and may serve for the development of new pharmaceuticals.

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IMMOBILIZATION OF NITROGEN-FIXING *BACILLUS FLEXUS STRAIN* BLY01 FOR ENHANCED SYNTHESIS OF α-AMYLASE AND ALKALINE PHOSPHATASE

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ABSTRACT

A nitrogen-fixing gram positive spore forming bacteria was isolated from potato waste and produced α-amylase and alkaline phosphatase (APase) when the organism had been fed on starch and insoluble $Ca_2(PO_4)_3$ in nitrogen free medium. The organism was identified as *Bacillus flexus* BLY01 on the basis of morphological as well as phylogenetic analysis using 16S rDNA sequencing. Under optimized conditions in broth culture, fresh cells produced about 56 unit/ml of α-amylase and 16.75 unit/ml of alkaline-phosphatase. The log phase cells of strain BLY01 had been immobilized in Ca-alginate gel capsules and were used for the semi-continuous production of the substrate specific enzymes individually. The immobilized cells showed about 27% and 126% enhancement in activity of α-amylase and APase, respectively. The immobilized system retained 80% - 84% of its initial efficiency after 3rd cycle of fermentation batches.
One unit of α-amylase activity = 10 µg of reducing sugar per ml per min from soluble starch and one unit of APase =10 µg of para-nitrophenol formed per ml per min at 37^{0}C at pH 8.0.

Keywords: α-Amylase, alkaline phosphatase (APase), *Bacillus flexus,* immobilization, 16rDNA sequencing

INTRODUCTION

The application fields of microbial extracellular malto-oligosaccharide forming α-amylase and alkaline phosphatase (APase) are extremely wide and variable. The α-amylase (EC 3.2.1.1), which is mainly used as a thinning agent in starch hydrolysis, is widely applied in food, paper, textile, and pharmaceutical industries (**Pandey *et al.*, 2000; Konsoula and Liakopoulou-Kyriakides, 2004; Yang *et al.*, 2005**). APase (orthophosphate monoester phosphohydrolases, EC 3.1.3.1.) is the metalloenzyme, nonspecifc, phosphomonoesterases. Phosphatases are the most crucial enzymes for survival of organism that hydrolyze phosphate esters and provide inorganic phosphate (Pi). APase has wide range of applications in the field of disease diagnosis, biotechnology, immunology and molecular biology and serve as biochemical marker for quantitative measurement of disease (**Muginova *et al.*, 2007**). This enzyme has also immense application in agricultural fields for mineralization of insoluble phosphates present in soil and introduced as fertilizers and pesticide (Šarapatka, 2003) Though these enzymes exist in various organisms from bacteria to mammals; however, the microbial enzymes are more appreciable due to their effectiveness, specificity, biocompatibility, biodegradability and also from the economic view point (**Singh Nigam, 2013**).
The technique of cell immobilization has become a common practice for biotechnologists and industrial microbiologists. It has several advantages over fresh cell cultivation from the view points of product separation, re-use of biocatalysts, prevention of washout, reduced risk of contamination and operational stability (**Tanaka and Kawamoto, 2006**). The high density of microbial cells in beads makes them more efficient to show the enzymatic activity and productivity under specific and controlled environment. In comparison to various types of immobilization, the entrapment method using calcium alginate is easier, non-toxic and inexpensive (**Goksungur and Zorlu, 2001**).
The organism *Bacillus flexus* is known to synthesize α-amylase (**Zhao *et al.*, 2008**) and β- amylase (**Amano *et al.*, 2009**) but it has also been recorded as a producer of multiple enzymes like amylase, lipase and protease (**Tambekar and Dhundale, 2013**). Very few reports of APase synthesis by the *B. flexus* has been cited till date (**Patel and Sharma, 2012, 2013**). In the present study, cells of *Bacillus flexus* BLY01 has taken for calcium alginate immobilized bead preparation. The enzymatic efficiency of the fresh cells and immobilized cells to hydrolyze starch and *p*-nitrophenyl phosphate (*p*-NPP) has been investigated. The activities after 1st, 2nd and 3rd cycle of batch fermentation were compared, if any, at the same fermentation condition.

MATERIAL AND METHODS

Microorganisms, cultivation and storage

The organism *Bacillus flexus* BLY01 has been isolated from potato cold storage waste material collected from Bally, district Howrah, West Bengal, India. Enrichment culture following serial dilution and spread plate techniques the bacterial strain has been isolated on modified Stockdale nitrogen free medium having pH 7.7 (**Stockdale *et al.*, 1968**) containing starch, 1% (w/v) and calcium phosphate 0.2% (w/v) instead of glucose and dipotassium hydrogen phosphate, respectively. The organism was grown at 37^{0}C under shake flask condition (180 rpm) for 24h in the said broth medium and was stored on the agar slant at 4^{0}C.

Sequencing of 16S rDNA and identification of strain

Genomic DNA was extracted from the 24h grown culture using Genei Ultrapure TM Bacterial Genomic DNA Purification kit KT162 (Cat # 612116200021730). The universal primer sequence used for the amplification of 16s rDNA were: the Forward primer -- 5'-AGAGTTTGATCMTGGCTCAG -3' and the Reverse primer - 5' -TACGGYTACCTTGTTACGACTT-3'. Using primers, the ~1.5 kb 16S rDNA fragment was amplified using *Taq* DNA polymerase (3U). The final PCR mix was composed of genomic DNA: ~20ng; dNTP mix (2.5mM each): 1.0µl; Forward Primer: 100ng; Reverse Primer: 100ng; Taq Buffer A (10X): 1X; Taq Polymerase enzyme: 3U; $MgCl_2$: 25 mM; Glass distilled water: to make up the volume 50µl. The program for PCR was as follows: 94^{0}C for 5 min, 35 cycles of 94^{0}C for 30 Sec., 55^{0}C for 30 Sec, and 72^{0}C for 1.30 min, and extension at 72^{0}C for 10 min. Amplification was done using Perkin Elmer Gene Amp PCR system 2400. The PCR product was bi-directionally sequenced using the forward, reverse and an internal primer. Amplicons were visualized by electrophoresis on 1% agarose gel with StepUpTM 500bp DNA ladder (Cat# 612651970501730) and after staining with ethidium bromide. The Sequence data was aligned and analyzed for finding the closest homologous microbes using combination of the database of National Center for Biotechnology Information (NCBI) GenBank and ribosomal database project (RDP) website.

Nucleotide sequence accession number

The 16S rDNA partial sequence of the strain *B. flexus* BLY01 was deposited in the NCBI Gene Bankit nucleotide sequence database under accession number KF000098.

Determination of growth and cell number in bead

The growth of the organism was determined following the total count method using a haemocytometer and the cell number present in bead was confirmed after bursting of beads following the viable cell count method on fresh agar plate. The starch phosphate broth was inoculated with growing BLY01 cells of 12h at 4% (v/v) level and was incubated up to 35 h at 37^{0}C under shake flask condition (140 rpm) for fermentation.

Immobilization of cells

The fresh cells were harvested aseptically by centrifugation (10,000 rpm) at 4 ^{0}C and taken for immobilization following the method of **Kierstan and Bucke (1977)** with some modification. About 100 mg of fresh biomass was well mixed to the 50 ml of sodium alginate solution (4%, w/v). The mixture obtained was extruded drop wise through 0.1 ml fine tip pipettes into a 100 ml sterile $CaCl_2$ solution of 0.1M, used as cross linking agent. Alginate drops (approx. 2mm-3mm) were solidified in contact with $CaCl_2$ and thus entrapping the bacterial cells. Repeated washing and incubation in the same solution at 10^{0}C made the beads more hard and stable. Finally, the beads were washed with sterile saline to remove excess calcium ions. After determination of beads volume (approx. 0.01 - 0.16 cc) it was found that average bead contained about 0.02 - 0.03 mg of biomass.

Fermentation with immobilized cells

Fresh immobilized beads prepared following the above method contained approximately 0.02 mg of biomass each (calculated from the total volume of gel mixture and the amount of cells added to it) were suspended in starch-buffer solution (pH 8.0) for amylase activity determination. The phosphatase enzyme activity of immobilized cells was determined in glycine- NaOH buffer of pH 8.0 (pH optima for the enzyme). Beads were incubated for different time period and for 1-3 time cycles at 37 ^{0}C.

Analytical methods

Determination of extracellular amylase activity

The amylase enzyme activity was detected following chemical estimation of reducing sugar. The sugar produced in culture filtrate was assayed using di-nitrosalicylic acid reagent (**Miller, 1959**). Activity of the enzyme produced by the strain, fresh cells and immobilized cells was measured in units (U). One unit of α-amylase activity is defined as the amount of enzyme that releases 10 µg of reducing sugar per ml per min from soluble starch at pH 8.0.

Determination of extracellular APase activity

For determination of the extracellular enzymatic activity in the respective fermentation set, the supernatant was assayed calorimetrically (Systronics-166) following the method of Bernt (Bernt, 1974) in glycine-NaOH buffer (pH 8.0) at 37^{0}C using the substrate di-sodium salt of *para*- nitrophenyl phosphate (*p*-NPP), Sigma USA. One unit of phosphatase =10 µg of para-nitrophenol (*p*-NP) formed per ml per min at 37^{0}C.

RESULTS AND DISCUSSION

16S rDNA sequence similarity and phylogenetic analysis of the organism

Molecular identification of the isolated gram positive sporulating bacilli was carried out based on its 16S rDNA sequence analysis. The 1.5 kb sequences obtained from the strain (Fig.1) were aligned with all the presently available 16S rDNAsequences in the GenBank and RDP database. As a result, a phylogenetic tree was constructed using the neighbor joining method, and is shown in Fig.2. Phylogenetic analysis using the 16S rDNA sequences indicated that the organism was a strain of *Bacillus* and it had 99% 16S rDNA sequence-similarity with the *B. flexus* strain LF-3, strain SL21 and strain FFA4 (Table1).

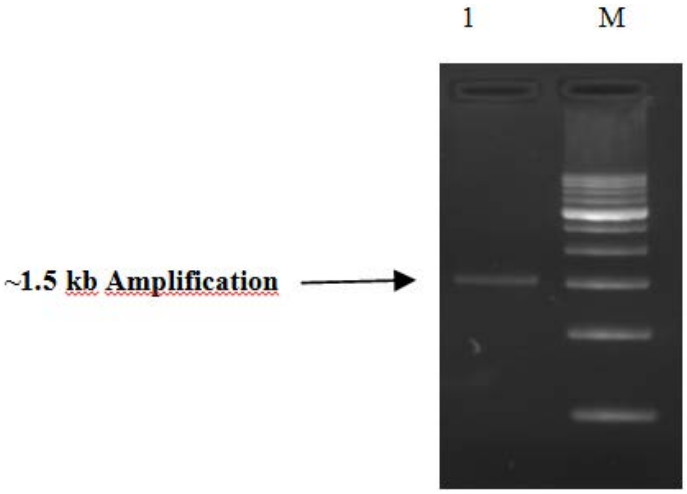


Figure 1 PCR amplification. The PCR products (~1.5 kb) were loaded on 1.0% agarose gel along with StepUp^{TM}500bp DNA ladder. Lane 1: PCR Amplification of 16srDNA sample from BLY01 and Lane M1: DNA ladder

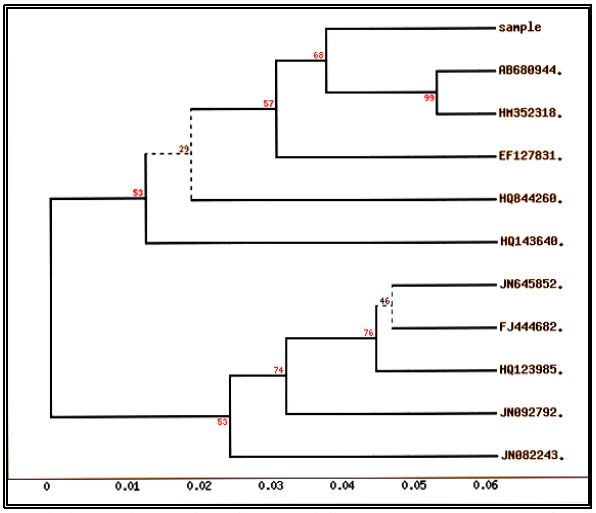


Figure 2 Phylogenetic tree based on partial 16S rDNA sequences, showing the relationship between the isolated strain BLY01 and other species belong to the genus *Bacillus*. The ID numbers of organisms are in parentheses. The tree was constructed using the neighbour-joining method. Numbers at nodes indicate levels of bootstrap support (%) determined from resampled data.

Table1 Alignment view of strain BLY01 using combination of NCBI GenBank and RDP database

Alignment View	ID	Alignment results	Sequence Description
	BLY01	1.00	Studied Sample
	HQ844260.1	0.99	*Bacillus sp. AS6*
	HQ143640.1	0.99	*Geobacillus stearothermophilus strain*
	EF127831.1	0.99	*Bacillus flexus strain LF-3*
	JN645852.1	0.99	*Bacillus flexus strain SL21*
	JN082243.1	0.98	*Bacillus sp. ce45*
	AB680944.1	0.98	*Bacillus flexus gene*
	JN092792.1	0.99	*Bacillus flexus strain FFA4*
	HM352318.1	0.98	*Bacillus sp. CmNA4*
	HQ123985.1	0.99	*Bacillus sp.*
	FJ444682.1	0.99	*uncultured Bacillus sp. clone 2y-54*

Growth and synthesis of enzymes

ime scale study of α- amylase activity of the BLY01 cells (Plate1) resulted in the accumulation of utilizable reducing sugar in the medium and enzyme increased steadily along with growth up to 25h of the incubation period and thereby decreased in the late phase of growth (Fig. 3). The maximum amylase synthesis (56.4 unit/ml) was shown by the stationary phase cells of the strain. Synthesis APase had started at an early stage of growth and continued to attain the maximum concentration of 16.5 unit/ ml after 30 h during the late stationary phase of growth, followed by a slow decline of the activity.

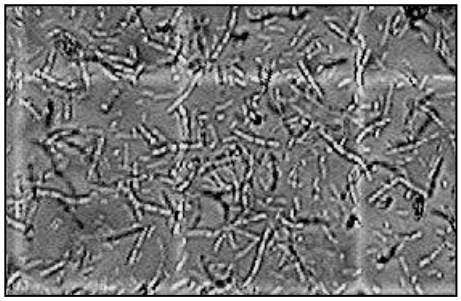


Plate 1 Cells of *B. flexus* BLY01 at their log phase before the onset of sporulation. Cells were grown in modified Stockdale medium under shake flask condition at 37^{0}C.

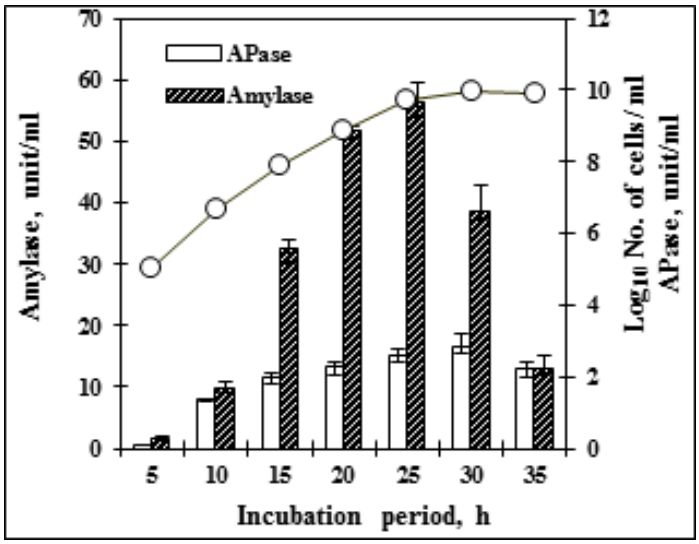
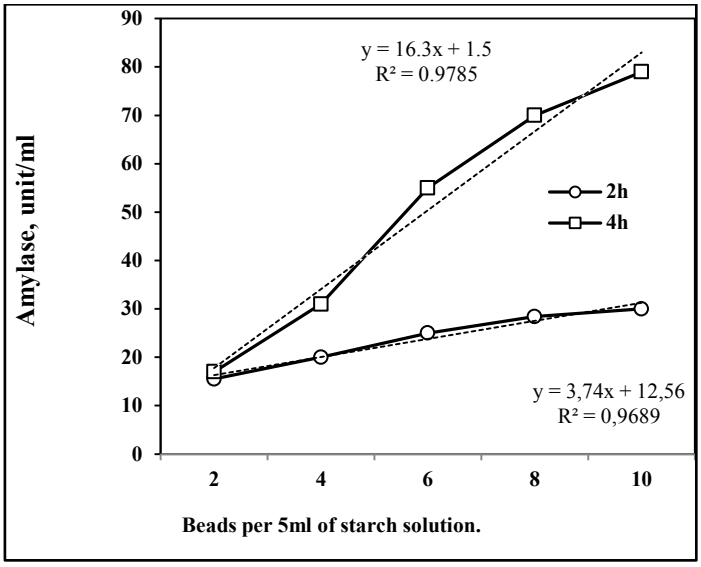


Figure 3. Time course depiction of growth as $\log_{10}$ number of cells per ml (-o-), α -amylase (▨) and APase (□) synthesis by *B. flexus*. BLY01 cells in starch–calcium phosphate medium under shake flask condition at 37^{0}C.

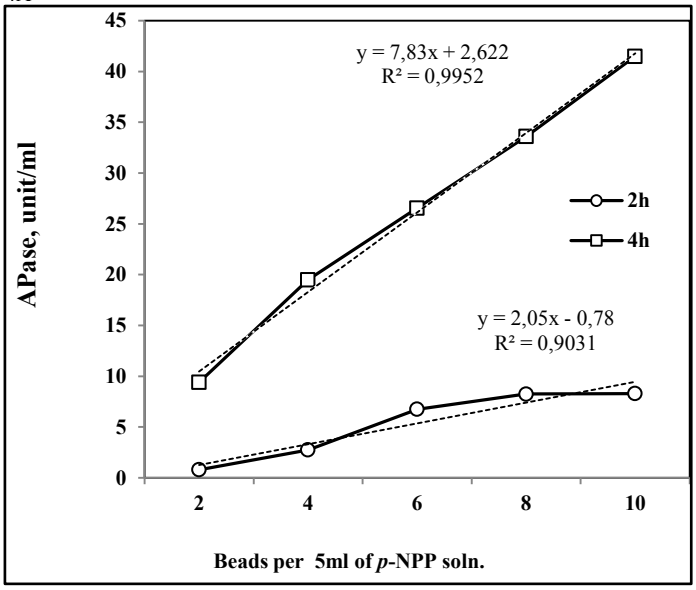
Fermentation with immobilized cells

For both of the amylase and Apase enzymes synthesis much quicker response (within 2 and 4 h) of immobilized cells (Plate2), compared to the fresh cells, were observed when the supernatant was taken for assay after beads incubation. Maximum enzymatic activities were found in the supernatants taken from 10 beads per 5ml fermentation sets (Fig.4 Aand B) *i.e.* 79 units and 41.5 units of amylase and APase respectively in per unit volume of reaction mixtures.

Plate 2 Fresh Ca-alginate beads containing *B. flexus* cells. Beads were prepared following modified method of Kierstan and Bucke (1977).



4A



4B

Figure 4A and 4B. Effect of beads number and incubation period on enzyme activities of *B. flexus* BLY01. Cells were immobilized in calcium alginate gel and incubated in starch solution at 37 ^{0}C under shake flask condition at 80 rpm. **Figure 4A** Amylase activity. **Figure 4B** APase activity

Repeated fermentation with Immobilized cells

The respective beads after a cycle of fermentation for 4 h were transferred aseptically to the fresh starch-buffer and to the *p*- nitrophenol phosphate buffer solution for determination of α- amylase and APase activity respectively and the process was repeated up to the third fermentation batch cycle considering it as the semi-continuous fermentation with immobilized cells of the strain BLY01. After each cycle of fermentation the efficiency of activity of either of the enzymes was reduced to some extent (Table 2). However, 84% and 80% of activity was retained in the immobilized cells for of α-amylase and APase, respectively, after 3rd fermentation cycle. Data from these incubations, however, were still higher than the activity of free cells of similar amount.

Table 2 Efficiency of free and immobilized cells of *B. flexus* BLY01 at different fermentation cycle.

Fermentation with	Amylase, unit/ml*	APase, unit/ml**
Free cells as high cell density culture (0.1 mg/5ml approx.)	56.8	16.5
Immobilized beads, 1st cycle	72.2	37.4
Immobilized beads, 2nd cycle	68.5	35.0
Immobilized beads, 3rd cycle	61.0	29.6

Legend: Beads were incubated and transferred aseptically. In each case 5 beads were incubated in 5 ml fermentation culture for 4 h at 37^{0}C under shake flask condition at 80 rpm.
*Amylase activity was determined following the method of **Miller (1959).**
APase activity was determined following the method of **Bernt (1974).

DISCUSSION

The gram-positive bacterium *Bacillus* is capable of secreting large amounts of endogenous proteins as enzymes into the extracellular medium. The strains of *Bacillus* have been reported as able producer of α-amylase (**Dey *et al.*, 2003; Kumar *et al*., 2012**) as well as APase enzyme (**Bookstein *et al*., 1990; Mahesh *et al*., 2010**) as their primary metabolites in presence of the respective substrates. It is evident that the secretion activity is rather low during organisms' exponential growth and increases substantially at the onset of stationary phase (**Priest, 1977**) which corroborates with the findings derived from the strain *B. flexus* BLY01 fresh cells (Fig.3). The sharp decline in the amylase activity by the strain BLY01 in its late phase had been depicted which might be due to the presence of a negligible amount of residual starch in the growth medium, compare to that of in the early phase of culture. Similarly, the data generated during determination of APase activity the strain BLY01 showed the repressive effect after the period when cells entered the stationary phase at 30th hour of incubation. It may happen due to the shifting of pH of the medium from alkaline to acidic (data not shown) as the results of accumulation of cellular metabolites and excess inorganic phosphate ions derived from APase activity.

The results of the amylase and APase activities by the cells of BLY01 entrapped in calcium alginate beads were found excellent. The same beads seemed to be the effective for either α- amylase activity or for APase activity. The proportional increment of enzyme activity with increase in immobilized bead number per volume of substrate-buffer solution had been noted. The activity of enzymes also increased with duration of incubation at 37^0C. Similar enhancement in amylase synthesis has been described using immobilized beads of *B. macerans* (Ahmed, 2008) and cellulose synthesis by immobilized *B. pumilus* EWBCM1 (**Kumar *et al.*, 2012**). More than 27% and 126% increments in the efficiency of α-amylase and APase respectively, were observed from the results obtained in the first cycle cultivation of immobilized BLY01 cells compare to those of same amount fresh cells activity. Similar enhancements in the amylase activity were described in case of *B. circulans* (**Dey *et al*., 2003**) and *B. subtilis* (**Konsoula and Liakopoulou-Kyriakides, 2006**) when cells were immobilized in Ca-alginate gel. However, in batch cultivation APase activity of the beads become limited in presence of inorganic phosphate ions which affect the integrity of calcium alginate beads (**Han *et al*., 2011**) unless the product is removed from the reaction site.

As the microbial enzymes amylase and APase as well have immence importance in food and pharcutical industries (**de Souza** and **de Oliveira Magalhães, 2010; Raja *et al.*, 2011; Christopher and Kumbalwar, 2015),** the technology of cell immobilization of *Bacillus flexus* BLY01 is aptly suited for dual enzymes synthesis using the same beads for quicker and vigor action.

CONCLUSION

The organism *Bacillus flexus* BLY01 thus proved to be a potent source for any of the enzymes α-amylase and APase which showed higher efficiency as entrapped cells in calcium alginate gels specially for APase activity and there was a minimum loss after 3rd cycle of fermentation. This type of reusable immobilized cells could be beneficial as stable and commercially viable biotechnological tool for biocatalyst activity.

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ANTIBACTERIAL ACTIVITY OF OZONIZED OLIVE (*OLEA EUROPAEA L.*) AND VENADILLO (*SWIETENIA HUMILIS* ZUCC.) OILS AGAINST *ESCHERICHIA COLI* AND *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Ozonized oils are antimicrobial agents obtained from the combination of ozone and unsaturated fatty acids of vegetables oils. The aim of the present study was to evaluate the antimicrobial effectiveness of ozonized olive oil (OOO) and ozonized venadillo oil (OVO) against *Escherichia coli* and *Staphylococcus aureus*. The antibacterial activity was conducted by the agar dilution method to determine the minimum inhibitory concentration (MIC) and the bacterial Log10 reduction. The lowest MIC (4.5 mg/mL) against *E. coli* was obtained when OOO and OVO were ozonized during 12 and 6 hours, with 2.5 Log10 of bacterial reduction, respectively; while, the lowest MIC against *S. aureus* (1.5 mg/mL) was obtained when OVO was ozonized during 6 hours, with 3.4 Log10 of bacterial reduction. The OOO reached peroxide values of 642.53 and 703.7 mmol-equiv/kg after 6 and 12 hours, respectively, while an 892.12 mmol-equiv/kg was obtained after 6 hours for OVO. Data reported here suggest that both ozonized oils are promising effective treatment for bacterial infections.

Keywords: Bactericide; Ozonized oils; Venadillo oil; *Escherichia coli*; *Staphylococcus aureus*

INTRODUCTION

In the past fifty years, the proliferation of antimicrobial agents for use in humans and animals has placed an unprecedented pressure on microorganisms. Thus, drug resistant bacteria have led to look for natural antibacterial products such as vegetables oils. Plants and their essential oils are potentially useful sources of antimicrobial compounds, such as phenolic acid, carvacrol, terpenes, terpenoids and geraniol (**Alonso-Castro *et al*., 2011; Association of Analytical Communities, 1969; Bakkali *et al*., 2008; Bassolé and Juliani, 2012; Burt, 2004**). The application of natural oils is wide, ranging from skin to periodontal infections; they had also been proposed for cancer treatment, food preservation, as well as aromatherapy and the fragrances industries (**Christaki et al., 2012**).
Another alternative for the treatment of infectious diseases is the use of ozone. Ozone is a powerful oxidizer, does not contaminate the atmosphere, possess an antimicrobial effect, and has not been reported with bacterial resistance. Ozone has been used against cutaneous infections, otitis, vaginitis, and dentistry interventions (**Criegee, 2003; Díaz et al., 2006; do Amarante et al., 2013**). Additionally, application of ozone on infections caused by *Escherichia coli* and *Staphylococcus aureus* had been reported (**Geweely, 2006; Guinesi et al., 2011; Jiménez et al., 1997**). The therapeutic antimicrobial properties of ozone are due to the formation of oxidized compounds, such as hydrogen peroxide, hydroperoxides, aldehydes and ozonides which are formed when the polyunsaturated fatty acids presents in vegetable oils make contact with ozone (**Kon and Rai, 2012**).
Over the last decade, many ozonized oils have been introduced as alternative for bacterial infections. Among those, ozonized sunflower oil (OLEOZON) and pure olive oil have been proven to have a broad antibacterial spectrum that covers Gram negative and Gram positive (**Geweely, 2006; Guinesi et al., 2011; Ledea et al., 2010; Lezcano et al., 1998**). The reaction between ozone and olive oil occur at the carbon-carbon double bonds present in unsaturated fatty acid producing different toxic products such as oxygenated compounds, hydroperoxides, ozonides, aldehydes, peroxides and polyperoxide which could be responsible for the wide antimicrobial activity of ozonized olive oil (**Kon and Rai, 2012**). **Díaz et al. (2006)** and **Lezcano et al. (1998)** assessed the antimicrobial activity of ozonized olive oil during 5 hours, showing a greater resistance against *E. coli* than *S. aureus* with a minimum inhibitory concentration of 9.5 mg/mL and 4.5 mg/mL, respectively. Venadillo tree (*Swietenia humilis Zucc.*) belongs to the Meliaceae family. It can be found along the Mexican and Central America Pacific coast. The secondary metabolites of venadillo tree mainly limonoids, represents a natural option to endemic microbial infections, which are traditionally used as infusions or ointments (**López-Pantoja et al., 2007; Martínez et al., 2006; Millezi et al., 2012**). **Lopez-Pantoja et al. (2007)** and **Montevecchi et al. (2013)** evaluated the antimicrobial activity of venadillo ethanolic extracts at 50% concentration, against *E. coli* and *S. aureus*, reporting the complete inhibition of growth of both microorganisms. The high content of polyunsaturated fatty acids of venadillo tree combines with ozone may potentiate the antimicrobial activity of the oil (**Martínez et al., 2006**). The objective of the present study was to evaluate the ozonized olive oil (OOO) and ozonized venadillo oil (OVO) against *Escherichia coli* and *Staphylococcus aureus*.

MATERIAL AND METHODS

Strains

Positive control strains of *Escherichia coli* ATCC 700609 and *Staphylococcus aureus* ATCC 29213 were obtained from the State Laboratory of Public Health of Sinaloa and the National Food Safety Laboratory Research, respectively. Both bacterial strains were used for the antimicrobial assays.

Ozone generation

Ozone was generated using the OzoneLabTM OL80F/DST-2S Desktop Ozone Generator (DST Lab, Canada) by passing Oxygen gas with an electric chamber at a fixed voltage (120 V) and a constant flow rate of 481 mg/hour.

Olive and venadillo oils preparation

Olive oil was commercially acquired and venadillo oil was obtained by seeds ethanolic extraction as described by **López-Pantoja et al. (2007)** and **Montevecchi et al. (2013)**.

Olive and venadillo oils ozonization

Ozonization was carried out during two periods of time, 6 and 12 hours for olive

oil, and 6 hours for venadillo oil. The venadillo oil was not ozonized during 12 hours because the peroxide value reached during 6 hours of ozonization was higher than those reached by ozonized olive oils. The ozone flow was 62 mL/minute with an output stream of 481 mg/hour. Additionally, commercially olive and venadillo oils were ozonized and used as standard for comparison. The resulted ozonized oils were named OOO and OVO for olive and venadillo, respectively.

Peroxide determination

The peroxide value of each sample was determined using the official methodology of AOAC 965.33 as followed: 0.5 g of each oil sample were placed in an Erlenmeyer flask with 30 mL of chloroform-acetic acid (3:2 v/v) (JT Baker) solution and stirred bar until dissolved. After this, 0.5 mL of a saturated potassium iodide (KI) solution was added and allowed to stand for 3 minutes. Subsequently, 30 mL of water were added to the flask followed by titration using 0.1 N sodium thiosulphate, until the color changed from yellow to light yellow. Finally, 0.5 mL of 1% starch solution was added and stirred to release the iodine from the chloroform layer (**Perez-Rubio *et al*., 2012**).

Inoculum preparation

Escherichia coli ATCC 700609 and *Staphylococcus aureus* ATCC 29213 were grown in tripticasein soy broth (TSB) (Bioxon, USA), and incubated for 24 hours at 37°C, separately. Each organism was purified by centrifugation at 13 080 g for 10 minutes at 4°C. The suspension was washed twice with 20 mL of phosphate monobasic buffer (KH_2PO_4), then adjusted with 5 mL of KH_2PO_4 buffer and refrigerated at 4°C (**Lopez-Pantoja *et al*., 2007**). Decimal dilutions were prepared to determine the final inoculums concentration of *E. coli* (1 x 10^4 CFU/mL) and *Staphylococcus aureus* (1 x 10^7 CFU/mL).

Minimum inhibitory concentration (MICs)

MICs were determined by the agar dilution method according to the National Committee for Clinical Laboratory Standardization (NCCLS) guidelines. All susceptibility tests were repeated three times. The prepared ozonized oils were previously sterilized for 1 hour using UV light (360 nm) and added in serial dilutions from 1 mg/mL to 10 mg/mL, per separate, to Mueller Hinton agar plates, to finally be dried out at room temperature in a laminar flow hood (Envirco, USA). Subsequently, an inoculum of the selected bacteria was widespread at the surface of the agar-ozonized oils mixture and incubated 24 hours at 37°C. After incubation, the Petri dishes were placed on a dark non-refracting surface and the MICs were recorded as the lowest concentration of OOO and OVO inhibiting visible bacterial growth.

Bacterial log10 reduction

This procedure was conducted once the MIC's values were determined for each ozonized oil and each concentration as followed: 10 mL of each prepared TSB ozonized oil mixture was inoculated with *E. coli* at 1 x 10^4 CFU/mL and *S. aureus* at 1 x 10^7 CFU/mL followed by incubations for 24 hours at 37°C. After the incubation period, decimal dilutions were prepared from each concentration and plated on m-FC agar (m-FC) (DIFCO, USA) and Mannitol Salt Agar (MSA) (DIFCO, USA) for *E. coli* and *S. aureus*, and incubated at 45 and 37°C for 24 hours, respectively. Finally, the colony forming units (CFU) were quantified in a colony counter (SOL-BAT brand), model Q-20. Three replicas were made for each test procedure and bacterial counts were logarithmically transformed in order to calculate bacterial Log10 reduction using the following equation according to **Jimenez et al. (2010)**:

(Log10 initial count - Log10 final count) = Bacterial Log10 reduction

Data analysis

Analysis of variance (ANOVA) was performed using Bacterial Log10 reduction as response variable. Tukey test was used to determine differences between bacteria, ozonized oils and ozonization time with a significant level of ≤ 0.05. Data were subjected to MINITAB 15 (2007) for statistical analysis.

RESULTS AND DISCUSSION

Peroxide value

The lowest peroxide value of 642.53 mmol-equiv/kg was recorded when olive oil was ozonized for 6 hours; however, peroxide values of 703.7 mmol-equiv/kg were obtained at 12 hours. On the other hand, the highest peroxide value of 892.12 mmol-equiv/kg was recorded when venadillo oil was ozonized 6 hours (Table 1).

Table 1 Determination of peroxide value for ozonized Olive and Venadillo oils

Oils	Ozonization Time (h)	Peroxide Value*
Olive	6	642.53
	12	703.7
Venadillo	6	892.12

* Expressed in mmol-equiv·kg^{-1}

Minimum inhibitory concentration (MICs)

When olive oil was ozonized 6 hours, the MIC's values for *E. coli* and *S. aureus* were 8.5 mg/mL and 3 mg/mL, respectively. Olive oil ozonized during 12 hours showed MIC's values of 4.5 mg/mL and 2.5 mg/mL for *E. coli* and *S. aureus*, respectively. Ozonized venadillo oil at 6 hours reported MIC's values of 4.5 mg/mL and 1.5 mg/mL for *E. coli* and *S. aureus*, respectively (Table 2).

Table 2 Antimicrobial activity of ozonized oils

Microorganisms	Olive oil				Venadillo Oil	
	6 h*		12 h*		6 h*	
	MIC†	$Log_{10}R$‡	MIC†	$Log_{10}R$‡	MIC†	$Log_{10}R$‡
Escherichia coli	8.5	3.3	4.5	2.5	4.5	2.8
Staphylococcus aureus	3	3.7	2.5	4.0	1.5	3.4

* Ozonization time
† Minimum inhibitory concentration expressed in mg·mL^{-1}
‡ Bacterial Log_{10} reduction

Bacterial log10 reduction

Table 2 shows the mean values of bacterial Log10 reduction. The higher antimicrobial activity was observed when olive oil was ozonized during 12 hours against *S. aureus* (4 Log10). *S. aureus* showed a higher reduction than *E. coli* at any ozonized oils and time tested. ANOVA tests showed statistical differences in the response of bacteria against the ozonized oils (p=0.000). However, no statistical differences were observed when ozonized oils where compared (p=0.154) or ozonization time was evaluated (p=0.260) (Table 3).

Table 3 Effects of bacteria, ozonized oil and ozonization time over antimicrobial activity

Source	Sum-of-squares	Degree of freedom	*F*-ratio	*p*-level*
Bacteria	2.6473	1	22.42	0.000
Ozonized oil	0.1337	1	2.28	0.154
Time	0.1629	1	1.38	0.260
Error	1.6530	14		

*alpha ≤ 0.05 significance level

The primary target, when ozone is combined with vegetable oil are carbon to carbon double bonds of unsaturated fatty acids, favoring the formation of hydrogen peroxide as final product (**Lezcano *et al*., 1998**). Thus, the possible mechanism by which ozonized oil acts as an antimicrobial, is the oxidation of the microorganism through a slow release of peroxide (**Ran *et al*., 2010**). In previous studies, the ozonized olive oil (Oleozone) reported peroxide values between 500 and 800 mmol-equiv/kg, with optimal antifungal activity at 650 mmolequiv/kg (**Ledea *et al*., 2010**). Similar peroxide values (642 mmol-equiv/kg) were obtained with ozonized olive oil in the present study. However, the differences between Oleozone and the ozonized olive oil used in the present study were the ozonization method, and the time to reach the optimal peroxide level. The ozone bubbling method used to ozonize Oleozon is carried out during eight weeks and the electric shock field employed to ozonize the olive oil can be performed in hours. Even though, both procedures are easy to use, the ozonization time differs. In this sense, the electric shock method optimizes time without affecting the formation of oxidized compounds. Also, no statistical differences were observed, in the bacterial Log10 reduction when olive oil was ozonized during 6 and 12 hours; therefore, 6 hours ozonization time can be adopted to optimize the ozonification process without affecting the antimicrobial activity.

On the other hand, venadillo oil generated high content of peroxide compounds due to the high presence of unsaturated fatty acids, allowing the production of various oxygenated compounds such as peroxides, ozonides and aldehydes, throughout the Criegee mechanism (**Sechi *et al*., 2001; Solórzano-Santos and Miranda-Novales, 2010**) thus, increasing its antimicrobial activity. Additionally, the statistical analysis showed no differences between ozonized venadillo and olive oils, confirming the antimicrobial effectiveness of the ozonized venadillo oil. The antimicrobial evaluation of both ozonized oils showed greater efficacy against Gram (+) than Gram (-) bacteria (Table 2). Previous reports by **Diaz et al.** (**2006**) and **Lezcano *et al*.** (**1998**) documented that ozonized olive oil with

peroxide values of 735 mmolequiv/kg against *E. coli* ATCC 10536 and *S. aureus* ATCC 6538, showed MIC´s results of 9.5 mg/mL and 4.5 mg/mL, respectively. The results were better in this study, which can be due to change in the analyzed ATCC strain, different peroxide values and the ozonization procedure used. The MIC's results showed lower values when ozonized venadillo oil was evaluated against both bacteria. This could be related to the high value of peroxides formed in this oil; however, it is necessary to conduct specific studies to demonstrate the antimicrobial activity of venadillo active principles. **Lopez-Pantoja et al. (2007)** and **Montevecchi et al. (2013)** evaluated acetone, methanolic and ethanolic extracts of venadillo oil against *E. coli* and *S. aureus*; all extracts used at high concentrations (25 and 50%) and inoculum levels of 1 x 10^8 CFU/ml and 1.5 x 10^8 CFU/ml, respectively, were effective in controlling the growth of both bacteria, being *S. aureus* the most susceptible. Moreover, to our knowledge, the present study is the first report of evaluating ozonized venadillo oils against bacterial organisms (**Travagli *et al*., 2010; Zanardi *et al*., 2013**).

CONCLUSION

To date, threating of bacterial infections is a challenging task due to the appearance of resistance to antibiotics. Therefore the development of natural alternatives as a control agent is a focused of interest. Based on the results of the present study, it can be concluded that both ozonized venadillo and olive oils could be a natural alternative for treatment of bacterial infections. However, venadillo tree may represent a much better option because of its accessibility in the Mexican Pacific Coast and Central America. Nonetheless, additional studies are required to validate the potentiality of ozonized venadillo oil as an antibacterial agent in topical or oral applications.

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ISOLATION AND CHARACTERIZATION OF *Fusarium solani* CAUSING SOYBEAN SUDDEN DEATH SYNDROME IN KOREA

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ABSTRACT

Soybean sudden death syndrome (SDS), caused by members of the *Fusarium solani* species complex, is an important disease leading to substantial yield reductions. The threat of soybean SDS is becoming serious in Korea, probably due to changes in the climate favoring the prevalence of this disease. In this study, after isolating *Fusarium* spp. from root rot-symptomatic soybean, we determined that *F. solani* was a causal agent of soybean SDS based on morphological and molecular characterization as well as Koch's postulate. From 2009 to 2011, a total of nine isolates of *Fusarium solani* were collected from major soybean fields, Korea. Morphological and TEF-1α sequence analyses confirmed a variety of nine *F. solani* isolates. All *F. solani* isolates were close with *F. solani* species from Asia and mostly belonged to *F. solani f. sp. pisi* (SSLP14, 15, 16,19, and 20). Based on homology analysis of both ITS and TEF-*1α*, some isolates (SSLP2, 18, and 22) were similar to *F. solani* causing human eye keratitis, indicating a shared pathogenicity both on humans and plants. In a pathogenicity test, we observed hyphae in both xylem and phloem tissues from discolored roots and basal stems of plants exhibiting foliar SDS symptoms, indicating its contribution to foliar symptoms. Based on the differential reactions of soybean plant genotypes to *F. solani* isolates, one indigenous *F. solani* isolates, SSLP15 was identified having the highest levels of virulence. In addition, Danbaekkong and Jinpumkong 2 soybean were found to be resistant to SDS as demonstrated by slight symptom with less than 20% foliage affected. The evaluation of SDS resistance could be beneficial to support varietal improvement through extensive soybean breeding program in Korea.

Keywords: *Fusarium solani*, soybean, sudden death syndrome

INTRODUCTION

Fusarium is a large genus encompassing a wide variety of fungal species. This genus is important to various industrial and agricultural sectors. Several members of the *Fusarium* species complex, such as *Fusarium proliferatum*, *F. fujikuroi*, *F. verticillioides*, *F. equiseti,* and *F. solani* (**Marasas *et al*., 2006; Naim *et al*., 2008**), may affect in diseases of several agricultural crops including rice, maize (**Hsuan *et al*., 2011**), and soybean (**Aoki *et al*., 2012**). *F. solani* was grouped into different *formae specialis* (f. sp.) and varieties (**Nirenberg, 1995**) based on host range. Using the sequences of 28S rDNA, internal transcribed spacer (ITS) rDNA, and transcription elongation factor (TEF)-*1α*, molecular identification has shown that isolates from the *F. solani* species complex can be classified into 50 sub-specific lineages (**O'Donnell, 2000**). *F. solani* (Mart.) Sacc. (teleomorph: *Nectria haematococca*) (Berk. & Br.), is considered to be an important phytopathogenic fungal agent of several crop diseases, such as root and fruit rot of *Cucurbita* spp., root and stem rot of pea, root rot of bean, and dry rot of potato, as well as sudden death syndrome (SDS) of soybean (**Zaccardelli *et al*., 2008**).
F. solani (Mart.) Sacc causing soybean SDS was recently described as *F. solani* f. sp. *glycines* to emphasize its host specialization (**Aoki *et al*., 2003**). Soybean SDS can be caused by diverse *F. solani* strains originating from different geographical regions and under different environmental conditions (**Aoki *et al*., 2005; Swoboda, 2010**). SDS is one of the major soil-borne disease that leads to significant yield losses. The severity of this disease depends on soybean cultivar susceptibility, cultural practices, and the presence of a conducive environment, such as cool/warm temperatures and high soil moisture (**Roy *et al*., 1997**). SDS which was first discovered in Arkansas in 1971, became wide spread in other states in the United States and in other regions of North and South America (**Rupe *et al*., 2001**). Moreover, SDS has become one of the most important soybean diseases in the top ten world soybean producing areas (**Mueller *et al*., 2002**), such as the United States, Canada, Brazil, and Argentina (**Wrather *et al*., 1997; Navi and Yang, 2008; Leandro *et al*., 2013**).
F. solani infection begins at the roots of soybean plants and eventually, severe SDS leads to defoliation. The typical SDS symptoms include scattered and interveinal circular or irregular spots on leaves. In the foliar phase, some leaves prematurely turn yellow or brown, while some plants remain green and do not exhibit foliar symptoms (**Hartman *et al*., 1997**). With the development of SDS symptoms, all of the leaf tissue becomes chlorotic and necrotic, with a mottled, mosaic pattern on the upper leaves (**Navi and Yang, 2008**). Flower and young pod abortion can take place in severely infected plants. Other root symptoms, such as root rot and crown rot, develop during or before the appearance of prominent leaf symptoms. Internal and/or basal stem and tap root vascular tissues appear discolored and necrotic, which are obvious symptoms of SDS (**Roy *et al*., 1997**).
SDS evaluation protocols have been developed for leaf and stem symptom severity using stem inoculation and soil infestation techniques. Soybean cultivars are initially evaluated for resistance to *F. solani* in the field, then greenhouse and chamber experiments are conducted. A number of soybean cultivars have been screened for SDS resistance (**Gray, 1996; Hartman *et al*., 1997; Navi and Yang, 2008; Aoki *et al*., 2012**). Qualitative and quantitative resistance have been reported using United States Department of Agriculture (USDA) germplasm collections and variants of regenerated plants, but the majority of modern cultivars are considerably susceptible to SDS (**Jin *et al*., 1996**). The virulence ability of *F. solani* isolates causing soybean SDS varies (**Li *et al*., 1999**), and a

preliminary report suggests the involvement of various races of *F. solani* in the severity of this disease (**Mueller *et al*., 2002**).

As current climate conditions change in Korea becoming more favorable for the development of SDS, isolating pathogenic *F. solani* and screening for soybean genotypes resistant to SDS are the first steps in a soybean breeding program aimed at controlling this disease. A number of indigenous *F. solani* strains from Korea was explored (**Gopal *et al*., 2012**) but not for SDS evaluation purpose. Therefore, the present work was undertaken to isolate and identify *Fusarium* species based on morphological and molecular approaches using internal transcribed spacer (ITS) and transcription elongation factor (TEF)-1α, notably *F. solani,* from diseased soybean plants with SDS-like symptoms or root rot. In addition, we assessed the virulence of *F. solani* isolates against different soybean genotypes under artificially controlled conditions.

MATERIAL AND METHODS

Collection and isolation of Fusarium species

Soybean plants with SDS-like symptoms which were characterized by discolored stems and root rot, were collected at the R5 to R6 growth stages from two different fields, in Suwon (latitude:37º17‘27‘‘N, longitude:127º00‘32‘‘E, elevation above sea level:58 m) and Daegu (latitude:35º52’13‘‘N, longitude:128º35’27‘‘E; elevation above sea level:45m), Korea, over a 3-year period, from 2009 to 2011. Both areas have similar long winter and high soil moisture. Suwon has the average rainfall of 1311 mm, average annual temperature of 11.6 ºC, and minimum and maximum temperature of -5 to-10 ºC and around 30 ºC, respectively. While in Daegu, the average rainfall is 1055 mm and the average temperature is 13.6 ºC, the average minimum temperature is -2 to -4 ºC while maximum temperature is around 30 ºC. These fields experienced continuous soybean cultivation. Small pieces (1–2 × 1–2 mm) of basal stems and roots were surface-sterilized in 1% sodium hypochlorite and rinsed in sterile water. The tissue sections were then air-dried on filter paper and plated onto potato dextrose agar (PDA) medium supplemented with 500 mg/L streptomycin to suppress bacterial growth. The culture plates were incubated at 25°C under a 12 h light/dark regime. The obtained isolates were purified by sub-culturing single spores, and the pure fungal isolates were stored in 30% glycerol at -80 °C until use.

Molecular and morphological characterization of *F. solani*

For genomic DNA isolation, each fungal strain was grown in liquid complete medium (CM) at 25 °C on a rotary shaker (150 rpm) for 3 days, and the mycelial mass was harvested and lyophilized. DNA was extracted with a cetyltrimethylammonium bromide protocol (**Leslie and Summerell, 2006**). All fungal isolates were first observed their macroscopic morphology. These isolates were then identified based on their sequences of internal transcribed spacer (ITS) region of ribosomal DNA amplified and sequenced with an ITS primer pair, ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') (**White *et al*., 1990**). *F. solani* species were further characterized by using DNA sequences of partial translation elongation factor (TEF)-1α amplified with specific primers ef1 (forward primer; 5'-ATG GGT AAG GA(A/G) GAC AAG AC-3) and ef2 (reverse primer; 5'-GGA (G/A)GT ACC AGT (G/C)AT CAT GTT-3' (**O’Donnell *et al*., 1998**). Oligonucleotides were synthesized by the Bioneer oligonucleotide synthesis facility (Daejon, South Korea). The amplified PCR product showing a single band on agarose gel electrophoresis was purified and used as a template for sequencing using a BigDye Terminator Cycle Sequencing Kit V.3.1 (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed with an automatic sequencer, Model ABI 3730 (Applied Biosystems) and the resulting sequences were analyzed using SeqScape software v. 2.0 (Applied Biosystems). For homology analysis, the sequences were compared against the non-redundant sequence data using BLAST. Sequences of several *Fusarium* species in the public database were included for phylogenetic tree construction. A neighbor-joining (NJ) tree was constructed with MEGA 4.0 using the bootstrap method with 1,000 replications (**Tamura *et al*., 2007**).

The morphological differences among the fungal isolates of *F.solani* were characterized by examining several parameters (colony, conidia and conidiophores, perithecia, and chlamydospores). Fungal isolates were grown on PDA for 8 days to observe fungal colony. Morphology of conidia, conidiophores, chlamydospore, and perithecia was observed from 6-14-day-old carnation leaf-piece agar (CLA) cultures (**Leslie and Summerell, 2006**). CLA cultures were incubated at 25 °C under near UV light (wavelength: 365 nm; HKiv Import & Export Co., Ltd., Xiamen, China) to induce asexual and sexual reproduction. Differential interference contrast (DIC) images were obtained with a DE/Axio Imager A1 microscope (Carl Zeiss, Oberkochen, Germany).

Evaluation of SDS susceptibility

Five soybean genotypes that have been primarily used as parents for genetic mapping populations were selected for pathogenicity testing using nine *F. solani* isolates. In addition to the five genotypes, four genotypes of a USDA germplasm collection including SDS-resistant genotype (PI 536636: Ripley), SDS-susceptible genotype (PI 652935: BARC-19), as well as Haiiro (PI 243530) and Spencer (PI 525454) were also included against three selected *F. solani* isolates (SSLP2, 20, and 22).

F. solani isolates were grown at 25 ºC for 7 days and used to infest red sorghum seeds that had been soaked overnight and autoclaved twice. Five plugs (4 mm in diameter) of mycelia were used for inoculation. The seeds were incubated with the mycelia at 24 °C for 2 weeks. A mixture of sterilized fertilizer:soil (1:1) was placed into 10×10×10 cm^3 tubes. The drain hole of each tube was plugged with non-absorbent cotton, the tube was prefilled with sterile soil mixture, and 7 g of infected sorghum seeds were evenly distributed onto the soil mixture. Additional soil mixture was added to each tube, directly covering the infected seeds, at a depth of 2 cm. Soybean seeds were planted in the soil mixture, and the seeds were covered with additional soil mixture. Non-inoculated red sorghum seeds were used as a control. A pathogenicity test was conducted in a growth chamber programmed for 12 h day/night, a temperature of 25 °C, and a relative humidity of 80%, with three replications per treatment. After seedling emergence, the soil moisture was maintained near water holding capacity. Four weeks after planting, each plant was evaluated for SDS leaf symptoms using disease rating according to standard criteria (**Hartman *et al*., 1997**). The criteria were determined as follows: 1 = no symptoms (high resistant), 2 = slight symptom showing 1 to 20% foliage affected (resistant), 3 = 21 to 50% foliage affected (moderate), 4 = heavy symptom with 51 to 80% foliage affected (susceptible), and 5 = severe symptom showing 81 to 100% foliage affected (high susceptible).

Colonization of *F. solani* in soybean

The stems and roots of *F. solani*-inoculated plants showing SDS symptoms were collected 4 weeks after planting for observation via microscopy. The plants were rinsed in running tap water and prepared for microtome sectioning. Both *F. solani*-inoculated and control samples were fixed with modified Karnovsky’s fixative solution (2% paraformaldehyde supplemented with 2% glutaraldehyde in 0.05 mol/L sodium cacodylate buffer) and washed in 0.05 mol/L sodium cacodylate buffer. The samples were then post-fixed in 1% osmium tetroxide in 0.05 mol/L sodium cacodylate buffer and briefly washed twice in distilled water. The tissues were infiltrated with stain and embedded overnight by *en bloc* staining with 0.5% uranyl acetate at 4 °C. After the samples were dehydrated in a graded ethanol series and propylene oxide and embedded in Spurr’s resin, they were polymerized at 70 °C for 24 h. Thick sections were generated with an ultra microtome and observed by light microscopy.

Statistical analysis

All data were subject to analysis of variance (ANOVA) using SAS software (SAS, Cary, NC, USA). The statistical difference in infection rate was tested by the least significant differences (LSD) at $P \leq 0.05$ (**SAS, 2002**).

RESULTS

Isolation of *F. solani*

All fungal colonies grew rapidly (+ 9 cm in diameter) at room temperature, and macroconidia appeared one week after inoculation. Most isolates produced *Fusarium*-shaped conidia and possessed cottony aerial mycelia with various color from whitish, yellowish-white or pale to yellow, brownish, pinky to bluish, sometimes with purple were observed. A comparison of the genomic sequences of the ITS regions revealed that all isolates shared high sequence homology with known *Fusarium* species at a level ranging from 99 to 100% (Table 1). Of the 54 isolates examined, nine isolates were identified as *Fusarium solani*, and the rest were *F. equiseti* (31 isolates), *Gibberella moniliformis* (anamorph: *F. verticillioides*; 13 isolates), and *Gibberella zeae* (anamorph: *F. graminearum* Schwabe; one isolate). All *F. solani* were further characterized their molecular and morphological properties.

Molecular and morphological characterization of *F. solani*

TEF sequences of nine *F. solani* isolates (SSLP2, 14, 15, 16, 17, 18, 19, 20, and 22) with other *Nectria haematococca-F. solani* species isolated from various hosts were used for the phylogenetic analysis (**O'Donnell, 2000**). All of the strains identified in this study were included in the clade 3 which contained Asian *F. solani* species (Fig. 1). Five isolates (SSLP14, 15, 16, 19, and 20) were grouped with *F. solani* f. sp. *pisi* strains. SSLP2/22 and SSLP18 strains were similar in TEF sequences with *F. solani* strains causing human eye keratitis at USA and India, respectively (**O'Donnell *et al*., 2007**). SSLP17 was out grouped with *Neocosmospora africana* (another sexual stage name of *F. solani*) which was isolated from South African soil (**O'Donnell, 2000**).Based on the virulence level category, isolates with high virulence especially SSLP15, 14 and 20 tend to be the same subclade indicating their close genetic relationship to support their same environment in Daegu where this site is more favorable for SDS than

Suwon. While isolates having lower virulence seemed to distribute in several branches.

Fungal characters on PDA vary depending on strains (Fig. 2). Cultures of SSLP14/15/20, SSLP16/19, and SSLP18 strains were white to creamy with sparse mycelium, which is typical for most *F. solani* species (**Leslie and Summerell, 2006**). SSLP17 strain grew very slowly compared to other strains, and both SSLP2 and 22 produced violet pigments with fluffier aerial mycelium (Fig. 2).

F. solani produced asexual spores in the form of macroconidia and microconidia. Macroconidia were faintly curved with a notched basal cell of *F. solani* which usually produces 3- to 7-septate macroconidia and 0- or 2-septate microconidia (**Leslie and Summerell, 2006**). In our study, however, few microconidia were seen in among *F. solani* isolates. Cream sporodochia, lumps of macroconidium, were common on carnation leaf pieces in all of the *F. solani* strains and microconidia were observed on remained agar surface of CLA medium (Fig. 3A). Seven strains (SSLP14, 15, 16, 17, 18, 19, and 20) produced typically shaped macroconidia (Fig. 3B), phialide (Fig. 3C), and microconidia (Fig. 3D) for *F. solani*. However, SSLP2 and 22 strains generated relatively longer macroconidia (Fig. 3E) and shorter microconidia (Fig. 3G) compared to other strains, whereas morphology of phialides was indistinguishable (Fig. 3F). Typical chlamydospores were observed in cultures of all of the tested strains (Fig. 3H). Only three *F. solani* strains (SSLP2, 17, and 22) produced red/orange perithecia (Fig. 3I) which contain rosette asci (Fig. 3J). In particular, SSLP17 strain produced more perithecia and fewer conidia compared to other strains.

Pathogenicity of *F.solani*

All *F. solani* isolates were examined for their virulence on various soybean genotypes. The isolates produced various levels of typical SDS symptoms, such as interveinal chlorosis and necrosis on young leaves, when inoculated on soybean plants grown in a growth chamber. Typical SDS symptoms were observed on soybean leaflets and roots. Notably, the *F.solani* pathogen was re-isolated from surrounding area of the infected soybean plants and identified as the same species, demonstrating Koch's postulated.

After inoculation with the isolates SSLP2, SSLP20, and SSLP22, SDS-resistant 'Ripley' showed an average SDS severity value of 0.03%, while a value of 21.9% was observed in SDS-susceptible 'BARC-19' which was comparable with'Spencer' (21.4%). None of the tested soybean genotypes exhibited lower values of SDS severity than Ripley or higher values than BARC-19, which was especially high when the later genotype was infected with SSLP22 (48.23%). Even though a bit less, this severity rating using SSLP22 indigenous from Korea is in good agreement with the USDA examination that BARC has been categorized as susceptible genotype. Haiiro' considerably exhibited resistance to SDS, with mean severity value of 12.2% against the three isolates, respectively (Table 2). This result suggests that different geographical and environmental conditions contribute the distinct virulence of *F. solani* strains.

The average SDS severity of Korean soybean genotypes was examined in plants grown in a growth chamber 4 weeks after inoculation with *F. solani* (Table 3). The main effect that showed significant differences was the percent of leaflets with SDS symptoms ($P< 0.05$), which depended on the fungal isolate used for inoculation. SSLP15 showed the greatest pathogenicity, with levels of 24.0%, whereas SSLP17 had the lowest pathogenicity at only 1.3%. Moreover, the virulence levels of selected isolates which were surveyed against the USDA genotypes and Korean genotypes were relatively comparable levels (Table 2 and Table 3). Highly significant soybean genotype effects were also detected ($P< 0.05$) in terms of disease severity. Danbaekkong and Jinpumkong 2 showed the lowest SDS severity (7.9%), while Pureunkong had the highest SDS severity (15.0%; Table 3). Significant interaction effects were also observed between isolates and soybean genotypes ($P< 0.05$). Pureunkong infected with SSLP15 exhibited the most severe SDS symptoms on leaflets (46.3%). In addition to Pureunkong, SSLP15 had the strongest virulence when Taekwangkong was infected, and Jinpumkong 2 was more severely infected by SSLP20. These results suggest that the host plant genotype affects the response of symptomatic leaflets to inoculation with *F. solani*.

***F. solani* colonization in soybean roots**

In the current study, colonization of *F. solani* was observed in host plants exhibiting SDS symptoms. No hyphae were observed in non-inoculated or healthy plants suggesting no colonization of fungus in the plant tissue. But infected plants had external and internal discoloration of basal stems and taproots, representing SDS symptoms (Fig. 4) as the effect of *F. solani*. Hyphae were detected in both phloem (Fig. 4A) and xylem tissues (Fig. 4B) of discolored basal stems and roots, causing foliar-like SDS symptoms. We determined that xylem tissues were more effective as *F. solani* colonization zones than phloem (Fig. 4C), and xylem colonization contributed to foliar symptoms to a greater extent than phloem colonization, which supports observations of a previous study (**Navi and Yang, 2008**).

Table 1 List of *Fusarium* spp. isolates

Isolates	Species	Location	Year isolated	Homology (E-value)
SSLP1	*Fusarium equiseti*	Daegu	2010	100 (0)
SSLP2	*F. solani*	Daegu	2010	99 (0)
SSLP3	*F. equiseti*	Suwon	2011	100 (0)
SSLP8	*F. equiseti*	Suwon	2009	100 (0)
SSLP10	*Gibberella moniliformis*	Suwon	2009	100 (0)
SSLP11	*F. equiseti*	Suwon	2009	100 (0)
SSLP14	*F. solani* f. sp. *pisi*	Daegu	2010	100 (0)
SSLP15	*F. solani* f. sp. *pisi*	Daegu	2010	100 (0)
SSLP16	*F. solani* f. sp. *pisi*	Daegu	2010	100 (0)
SSLP17	*Neocosmospora african*	Daegu	2010	100 (0)
SSLP18	*F. solani*	Daegu	2010	100 (0)
SSLP19	*F. solani* f. sp. *pisi*	Daegu	2010	100 (0)
SSLP20	*F. solani* f. sp. *pisi*	Daegu	2010	100 (0)
SSLP22	*F. solani*	Daegu	2010	100 (0)
SSLP28	*G. moniliformis*	Suwon	2010	99 (0)
SSLP29	*G. moniliformis*	Suwon	2010	100 (0)
SSLP30	*G. moniliformis*	Suwon	2010	100 (0)
SSLP31	*F. equiseti*	Suwon	2010	100 (0)
SSLP32	*F. equiseti*	Suwon	2010	100 (0)
SSLP33	*F. equiseti*	Suwon	2010	100 (0)
SSLP34	*F. equiseti*	Daegu	2010	100 (0)
SSLP35	*F. equiseti*	Daegu	2010	99 (0)
SSLP36	*G. moniliformis*	Daegu	2010	100 (0)
SSLP37	*G. moniliformis*	Daegu	2010	100 (0)
SSLP38	*F. equiseti*	Suwon	2010	100 (0)
SSLP39	*F. equiseti*	Suwon	2010	100 (0)
SSLP40	*F. equiseti*	Daegu	2010	100 (0)
SSLP41	*F. equiseti*	Daegu	2010	100 (0)
SSLP42	*F. equiseti*	Daegu	2010	100 (0)
SSLP43	*F. equiseti*	Suwon	2009	100 (0)
SSLP44	*F. equiseti*	Suwon	2009	100 (0)
SSLP45	*F. equiseti*	Suwon	2009	100 (0)
SSLP46	*F. equiseti*	Suwon	2009	100 (0)
SSLP47	*F. equiseti*	Suwon	2011	100 (0)
SSLP49	*G. moniliformis*	Suwon	2011	100 (0)
SSLP50	*G. moniliformis*	Suwon	2011	100 (0)
SSLP51	*G. moniliformis*	Suwon	2011	100 (0)
SSLP52	*G. moniliformis*	Suwon	2011	100 (0)
SSLP53	*G. moniliformis*	Suwon	2011	100 (0)
SSLP54	*G. moniliformis*	Suwon	2011	100 (0)
SSLP55	*G. moniliformis*	Suwon	2011	100 (0)
SSLP56	*F. equiseti*	Suwon	2011	99 (0)
SSLP58	*F. equiseti*	Suwon	2011	100 (0)
SSLP60	*F. equiseti*	Suwon	2011	100 (0)
SSLP61	*F. equiseti*	Suwon	2011	100 (0)
SSLP62	*F. equiseti*	Suwon	2011	100 (0)
SSLP63	*F. equiseti*	Suwon	2011	100 (0)
SSLP64	*F. equiseti*	Suwon	2011	100 (0)
SSLP67	*G. zeae*	Suwon	2011	100 (0)
SSLP68	*F. equiseti*	Suwon	2011	100 (0)
SSLP69	*F. equiseti*	Suwon	2011	99 (0)
SSLP71	*F. equiseti*	Suwon	2011	100 (0)
SSLP74	*F. equiseti*	Suwon	2011	100 (0)
SSLP75	*F. equiseti*	Suwon	2011	100 (0)

Fusarium solani species and its asexual stage are denoted in bold letters

Table 2 Mean SDS severity values for USDA soybean genotypes 4 weeks after inoculation with *F. solani* isolates (SSLP2, 20, 22)

Isolates	Soybean genotypes				Mean*
	BARC-19	Ripley	Spencer	Haiiro	
	Severity rating (%)**				
SSLP2	8.18	0	9.81	5.20	5.80^{c}
SSLP20	9.18	0	42.22	9.26	15.16^{b}
SSLP22	48.23	0.10	12.21	22.07	20.65^{a}
Mean*	21.9^{a}	0.03^{d}	21.4^{b}	12.2^{c}	

*Within fungal inoculum and host soybean genotype combinations, means in column followed by the same letter are not significantly different at $P \leq 0.05$ based on LSD

**Categories of SDSseveriy rating: 1 = no symptoms (high resistant), 2 = slight symptom showing 1 to 20% foliage affected (resistant), 3 = 21 to 50% foliage affected (moderate), 4 = heavy symptom with 51 to 80% foliage affected (susceptible), and 5 = severe symptom showing 81 to 100% foliage affected (high susceptible)

Table 3 SDS severity of host soybean plant genotypes against *F. solani* isolates

Isolates	Soybean genotypes					
	Danbaekkong	Pureunkong	Taekwangkong	SS2-2	Jinpumkong 2	Mean*
	Severity rating (%)**					
SSLP2	13.2	5.5	5.2	3.2	6.2	6.6^{cd}
SSLP14	5.4	43.4	14.3	26.4	5.0	18.9^{ab}
SSLP15	1.2	46.3	36.0	23.4	13.2	24.0^{a}
SSLP16	12.6	18.4	0.5	9.5	0.8	8.3^{c}
SSLP17	0.7	4.1	1.1	0.5	0.2	1.3^{d}
SSLP18	8.1	5. 5	26.3	0.5	0.2	8.1^{c}
SSLP19	0.3	19.3	11.3	0.7	0.7	6.4^{cd}
SSLP20	8.2	15.4	5.2	23.4	34.3	17.3^{b}
SSLP22	21.8	10.2	5.6	8.2	10.3	11.2^{c}
Mean*	7.9^{c}	15.0^{a}	11.7^{b}	10.6^{ab}	7.9^{c}	

*Within fungal inoculum and host soybean genotype combinations, means (column or row) not followed by the same letter are significantly different at $P \leq 0.05$ based on LSD, **The categories of SDS severity rating are the same as those on Table 2

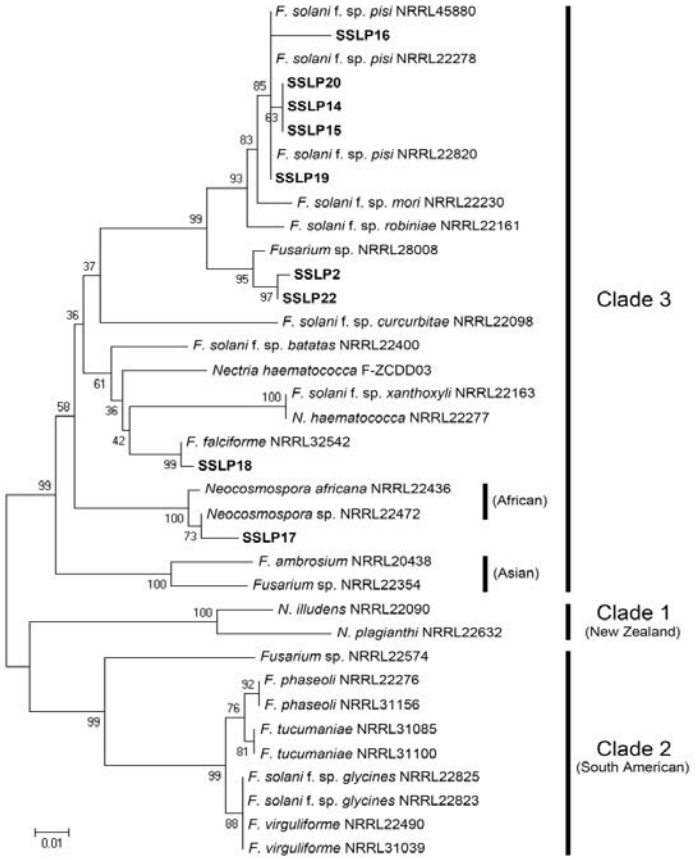

Figure 1 Phylogenetic tree of *F. solani* species complex produced by examining the sequence homology of the partial translation elongation factor (TEF)-1α using the neighbor-joining method with bootstrap values from 1000 replications

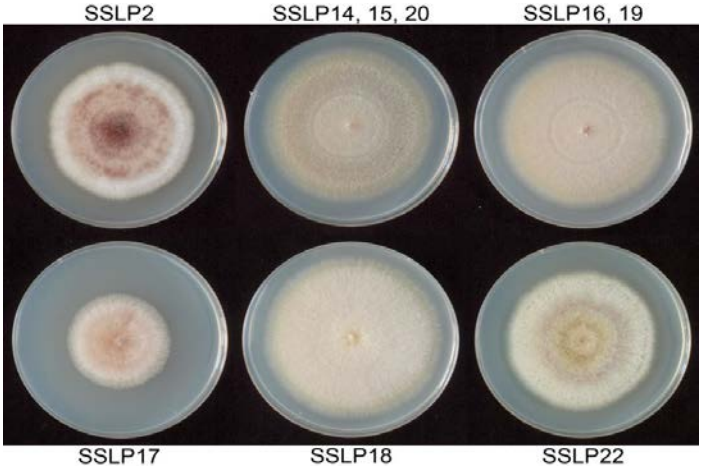

Figure 2 Mycelial growth of *F. solani* strains on potato dextrose agar (PDA) incubated at 25ºC. Pictures were taken 8 days after inoculation on PDA

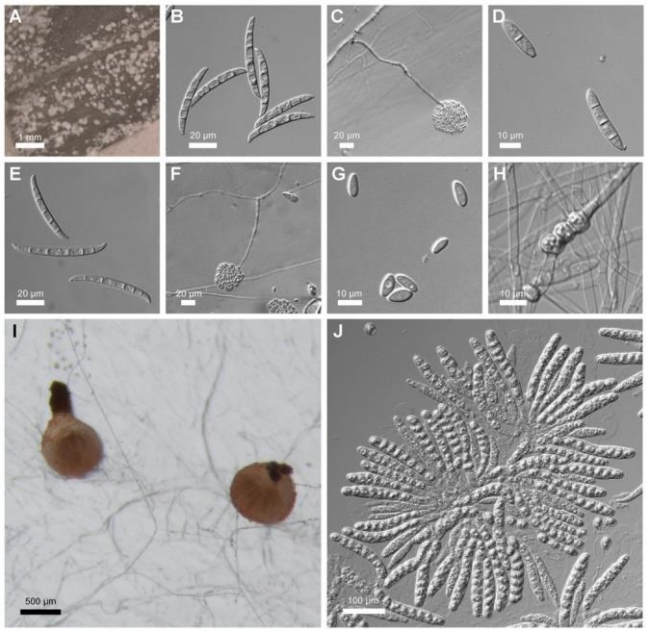

Figure 3 Morphological characters of *F. solani* strains. **A**, sporodochia produced on carnation leaf pieces. Macroconidia (**B**), phialide (**C**), and microconidia (**D**) produced by SSLP14, 15, 16, 17, 18, 19, or 20 strains. Macroconidia (**E**), phialide (**F**), and microconidia (**G**) produced by SSLP2 or 22 strains. **H**, chlamydospores produced by *F. solani* strains. **I**, red/orange perithecia produced by SSLP2, 17, or 22 strains. **J**, rosette asci dissected from perithecia. Pictures were taken 6-14 days after inoculation on carnation leaf agar (CLA) cultures

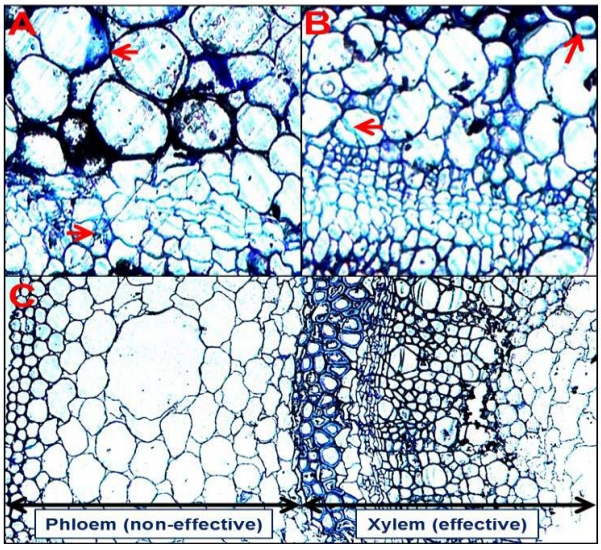

Figure 4 Colonization of *F. solani* hyphae in cross sections of tissue from a soybean plant with foliar, basal stem, and root symptoms of SDS. **A**, *F. solani* hyphae in phloem tissue. **B**, *F. solani* hyphae in xylem tissue. **C**, Colonization of *F. solani* in effective and non-effective zones in the xylem and phloem tissues of soybean, respectively

DISCUSSION

Sudden death syndrome (SDS) is thought to be optimal in favored high soil moisture and low temperature during the early growing season of soybean followed by relatively high temperature. Longer winter and higher rainfall with hot climate during summer seem to attribute the occurrence of SDS and root rot symptom soybean crop in Korea recently. As demonstrated in this current study, during 2009-2011, SDS-like symptom with root rot was observed in some fields of cultivated soybean in Korea, leading to isolate the fungal pathogen.

We identified predominant species of *Fusarium,* especially *F. solani*, *F. equiseti*, *G. moniliformis*, *F. solani*, and *G. Zeae* based on molecular and morphological approaches. Morphological observation is helpful to identify this Fusarium species because literally, the morphological characteristics assisted to initially differentiate fungal species even though easy misidentification usually occurred. Morphological identification of *Fusarium* species (**Marasas *et al*., 2001; Hsuan 2011**) is able to sort important species especially *F. solani* group before employing another method (**Leslie and Summerell, 2006**) or molecular approach. In particular, the identified *F. solani* from SDS-diseased soybean plants agreed with previous studies that this fungus causes soybean SDS. These suggest that cool soil temperatures and higher humidity levels during the 3-year sampling period, have a favorable environment for SDS pathogen development. Moreover, these fields contain historical hot spots for soybean diseases, with continuous soybean planting. This condition can be more severe since some plant-parasitic nematodes are interacted with SDS development (**Xing and Westphal, 2013**), however, *F. solani* was also reported to be pathogenic to the nematode eggs of *Heterodera. glycines* (**McLean and Lawrence, 1995**). In addition, *F. equiseti* and *G. moniliformis* species complex are the most common diseases reported in agricultural crops worldwide, including soybean (**Jasnic *et al*., 2005; Hsuan *et al*., 2011**). Recently, *F. graminearum* was also reported to be pathogenic and a causal agent of SDS in cereal crops as well as soybean (**Martinelli *et al*., 2004; Brar *et al*., 2011**).

Based on the criterion of the morphology of microconidia produced on the conidiophore and macroconidia formed on sporodochia, nine *F. solani* in our study showed typical characters (Fig. 3). These morphological characteristics could be a basic information to classification into formae specialis of fungal isolates. Morphological characters of some isolates were matched with *F. solani* f. sp. *pisi* described previously (**Jung *et al*., 1999**). These initial morphological characters are very potential for genetic studies on the molecular basis of pathogenicity. Moreover, part of ascospore could be seen clearly among isolates, might be responsible for their survival as shown by their morphological structure.

Phylogenetic tree analysis revealed that a variety of soybean SDS-causing *F. solani* strains were found in Korea. All isolates seem genetically closer to the *F. solani* from Asia than other continents. This SDS agent, *F. solani* isolates from Korea could be specific because they had far distance with F. *solani* species complex, such as *F. tucumaniae* and *F. virguliforme* from South America which are well known as SDS agent as well. A number of isolates (SSLP14, 15, 16, 19 and 20) which belong to *F. solani* f. sp. *pisi* suggests that the habitat of this species varies, not only pea, ginseng, mulberry, chickpea (**Matuo and Snyder, 1973**) but also soybean. As supported by a prior study that *F. solani* f. sp. *pisi* was also found on soybean and showed remarkable virulence in pea seedling (**Jung *et al*., 1999**). Another finding showing one of a sexual stage of *F. solani*, *Neocosmospora africana* (SSLP17) was also demonstrated. These asexual and sexual behaviors may be useful for deeper identification of formae speciales and races of these pathogens. While, some isolates (SSLP2/22 and SSLP18) similar to *F. solani* causing human eye keratitis indicate that the results are consistent with a claim that some *F. solani* species shares pathogenicity both on humans and plants (**Zhang *et al*., 2006**). Even though human eye keratitis caused by *F. solani* can be established fairly slow, but this could progress rapidly (**Wu *et al*., 2004**). Further studies should be conducted to these particular isolates to investigate fusarial keratitis infection on eye using mice model.

To help elucidate the mechanism of root infection by *F. solani*, it is important to relate aboveground symptoms to root colonization (**Navi and Yang, 2008**). Damaged tissues and discolored basal stems and taproots have hyphae in their xylem and phloem, indicating that infection of *F. solani* in root tissues causes the occurrence of foliar SDS symptoms. This result is in good agreement with the results of a previous study, which demonstrated that superficial colonization on taproots outside of the xylem produces fewer foliar symptoms than infection that occurs later in the growth stage (**Yang and Navi, 2003**). Moreover, early infection at the seedling stage enables the effective development of SDS fungi in xylem tissues, leading to foliar SDS symptoms (**Gao *et al*. 2006**). The current study demonstrates that the presence of SDS fungi in root tissues is associated with foliar symptoms, which supports a previous study showing a strong association between foliar expression of SDS and the incidence of root colonization (**Luo *et al*., 1999**). These foliar symptoms are predicted to be induced by a specific toxin(s) from *F. solani* (**Navi and Yang, 2008; Brar *et al*., 2011**) such as an acidic protein named FvTox1 which has been hypothesized to interfere with photosynthesis and causes foliar SDS. The way of penetration of fungi into plant tissue with its specific toxin movement may give a clue to the mechanism involved in the soybean host-pathogen interaction (**Pudake *et al*., 2013**).

F. solani can survive for several years by introducing its spores and mycelium into the soil and into soybean plants (**Aoki *et al*., 2003**). An SDS outbreak may occur after fungal mycelia become established and well-adapted to a new, favorable environment (**Malvic and Bussey, 2008**). In Korea, the soybean SDS occurrence has been increasingly surveyed, however, no studies prioritize the SDS evaluation. *F. solani* isolates were only found in Daegu, indicating that Daegu located in "basin" region with cool soil temperature and higher humidity gives a favorable environment for SDS pathogen development. The Daegu fields having a historical hot spot of soybean diseases with continuous soybean planting during several years were preferentially targeted for sample collection. As a result, fungi isolated from plants was affected by the nature of the diseased tissues. In this regard, geographical range is a critical factor to understand SDS and contributes a risk of soybean production in a region (Malvic and Bussey, 2008). Therefore, in soybean breeding programs, it is important to identify *F. solani* isolates with high pathogenicity and host plant genotypes with strong SDS resistance. In this study, the *F. solani* isolates SSLP15 showed the greatest virulence. These selected indigenous *F. solani* isolates having high pathogenicity may represent important fungal pathogen resources that can be used in the evaluation of soybean genotypes resistant to SDS in Korea. In addition to their pathogenicity assay, the examination of the *F. solani* population being as interaction or individual depending on species could be beneficial methods in SDS control management (**Marburger *et al*., 2014**).

Resistant varieties are one of the way to reduce the risk of yield losses due to SDS (**Leandro, *et al*., 2013**), thus it is necessary to incorporate SDS resistance into future soybean cultivars with increased yields (**Brzostowski *et al*., 2014; Adee, 2015**). In this study, we observed differential reactions of host soybean plant genotypes to *F. solani* isolates (Table 3). We selected five Korean soybean elite cultivars showing insect resistance (**Li *et al*., 2008**) and mostly used as mapping parents to support a good genetic material for pathogenicity test. None of the soybean genotypes showed high resistance to all *F. solani* isolates examined. However, Danbaekkong and Jinpumkong 2 were moderate resistant only to SSLP22 and SSLP20, respectively, and overall these genotypes exhibited resistance to SDS. Thus, Danbaekkong and Jinpumkong 2 may represent a good source of SDS resistance in addition to Ripley, which possess a single gene for resistance to *F. solani* based on the severity of leaf symptoms (**Gray, 1996**).

CONCLUSION

The characterized *F. solani* isolates showed their potency according to the pathogenicity and the highest pathogenic strain (SSLP15) could be useful to assist in the evaluation of soybean resistance to SDS. The selected genotypes resistant to SDS (Danbaekkong and Jinpumkong 2) could be used as resistance check/control for further screening of soybean genotypes for SDS resistance and genetic mapping of genes for SDS resistance which will be needed to successfully breed soybean using the virulent *F. solani* strains isolated in this study.

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EVALUATION OF ANTIOXIDANT AND CYTOTOXIC PROPERTIES OF CYANOBACTERIA, *LIMNOTHRIX* SP. AND *LEPTOLYNGBYA* SP. FROM ARABIAN SEA

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ABSTRACT

The hexane fractions of the marine cyanobacteria: *Leptolyngbya* sp. and *Limnothrix* sp., collected from Arabian Sea were found to display promising antioxidant properties than their ethyl acetate fraction during radical scavenging ABTS/DPPH assays (IC_{50}=30/330 and 60/260 µg ml^{-1} respectively). Phytochemical analyses of the fractions indicated presence of carbohydrates, flavanoids and steroids in both organisms. The hexane fraction of *Limnothrix* sp exhibited higher UV-Vis (300-450 nm) absorption, indicating presence of aromatic antioxidant compounds like flavanoids. This fraction was relatively non-toxic towards mouse fibroblast 3T3 cells (IC_{50} 400 µg ml^{-1}) and in fact, protected live cells from UV induced intracellular reactive oxygen species (ROS) stress. On the contrary, the hexane fraction of *Leptolyngbya* sp. had lower UV-Vis absorption, richer in alkaloids and also more cytotoxic (IC_{50} 200 µg ml^{-1}) towards the mouse fibroblast 3T3 cells. In short the hexane fraction of *Limnothrix* sp. is a safe antioxidant additive for food and healthcare products. On the other hand, the hexane fraction of *Leptolyngbya* sp. is rich in alkaloids and was cytotoxic, with potential application in cancer therapy.

Keywords: Marine cyanobacteria, Arabian Sea, antioxidant, UV, ROS, Cytotoxicity

INTRODUCTION

In recent years, marine cyanobacteria are being recognized as a potential source for novel antibacterial, anticancer (cytotoxic) and antioxidant molecules **(Dixit & Suseela, 2013)**. It is possible that antioxidant compounds are produced by these organisms as an adaptive response to mitigate the ill effects of high exposure to solar radiations. Within the solar UV radiations, the higher energy UV-B (315–280 nm) cause direct cellular damage while the lower energy UV-A (400–315 nm) act indirectly *via* reactive oxygen species (ROS) mediated pathway **(Armstrong & Kricker, 2001; Brash *et al*., 1991; Maltzman & Czyzyk, 1984)**. UV-A radiation convert intracellular triplet oxygen into reactive singlet state ($^3O_2 \rightarrow {}^1O_2$), which, then produce ROS through a cascade of intracellular energy transfer reactions **(Banks, Board, Carter, & Dodge, 1985; Hader, Kumar, Smith, & Worrest, 2007)**. Increased ROS levels lead to several harmful biochemical changes *viz*., lipid peroxidation, protein denaturing / damage, breakage of genetic materials **(Asok *et al*., 2012; Maisch, Bosl, Szeimies, Lehn, & Abels, 2005)** and even destruction of biochemical components vital for physiological metabolisms **(Halliwell, 1996)**. Antioxidants help organisms to mitigate ROS stress and protect food/cosmetics from oxidative decay **(Halliwell, 1996; Rastogi, Richa, Sinha, Singh, & HÃ¤der, 2010)**. Many of these compounds also prevent the progression of major human degenerative diseases **(Ames, Shigenaga, & Hagen, 1993; Takamatsu *et al*., 2003)**. The repertoire of natural antioxidants include polyphenols, flavonoids, vitamins, melatonin, pycocyanins and phlorotannins **(Chu, Lim, Radhakrishnan, & Lim, 2010; Gantar & Svircev, 2008; Kang *et al*., 2004; Pandey & Rizvi, 2009; Phang, Malek, & Ibrahim, 2013)**, while butylated hydroxy toulene (BHT), butylated hydroxy anisole (BHA) and tetra-butylated hydroquinones (TBHQ) are some of the synthetic compounds in market today. However, high cytotoxicity and lack of broad spectrum activity still limits their widespread usage**(Kahl & Kappus, 1993; Pandey & Rizvi, 2009)**.

Marine cyanobacteria are a rich source for several novel toxins, natural vitamins, carotenoids, phycobiliproteins, *etc*. **(Beltron & Nielan, 2000; Burja, Banaigs, Abou-Mansour, Grant Burgess, & Wright, 2001; Carmichael Wayne, Mahmood Nik, & Hyde Edward, 1990; Dixit & Suseela, 2013; Jha & Zi-rong, 2004)**. These organisms are ubiquitous in all niches of marine environments and contribute more than 70 % of the productivity of the open ocean **(Muhling *et al*., 2005)**. Nearly 300 alkaloids, constituting more than 24 % of all marine natural products used in biomedical research today, are of cyanobacterial origin **(Tan, 2013)**. Antioxidants constitute yet another group of promising metabolites from these organisms **(Takamatsu *et al*., 2003)**. Most of these compounds were reported from organisms belonging to the order Oscillatoriales, Nostocales, Chrococcales, Pleurocapsales and Stigonimatales **(Dixit & Suseela, 2013; Leao *et al*., 2013)**. Despite the abundance of bioactive metabolites, commercial exploitation of these organisms is still limited, mainly due to their slow growth rate in laboratory conditions and other practical problems associated with their mass culture **(Leao *et al*., 2013)**. Thus, though about 750 botanically identified cyanobacterial strains are known from Indian waters **(Van Baleen, 1962)**, systematic chemical investigation of only few of them have been accomplished till date **(Kosta, Jain, & Tiwari, 2010; Sundararaman, Averal, Akbarsha, & Subramanian, 1994)**. Here we report the phytochemical composition of two cyanobacterial strains, *Limnothrix* sp and *Leptolyngbya* sp from Arabian Sea and assayed *in vitro* antioxidant properties, cytotoxicity in mouse fibroblast 3T3 cells and ability to protect live 3T3 cells from UV induced reactive oxygen stress for the first time.

MATERIAL AND METHODS

Cyanobacterial isolates and culture condition

Filamentous cyanobacteria, *Limnothrix* sp. and *Leptolyngbya* sp., were isolated from surface waters of Arabian Sea in Artificial Seawater Nutrient –III (ASN-III) medium and were identified by morphological and molecular tools **(Nubel, Garcia-Pitchel, & Muyzer, 1997; Rippka, Deruelles, Waterbury, Herdman, & Stanier, 1979)**. The 16S rRNA gene sequences of the isolates are submitted to NCBI Genbank (JF4288391: *Limnothrix* sp. and KF793929: *Leptolyngbya* sp). The isolates were purified by serial dilution and maintained in ASN-III medium at room temperature with light: dark cycle of 12:12 hr (40 µmol photon m^{-2} s^{-1}

light). The purity of the culture was monitored regularly using optical microscopy and pH of the media was maintained at 7.5±0.5 using 0.1N HCl or NaOH.

Extraction of bioactive molecules

The cells were separated from a month old (i.e. stationary phase) culture by centrifugation at 5000 rpm for 10 min, and pellets were washed with Phosphate Buffered Saline (PBS). The crude extracts of cyanobacteria were prepared by extracting 10 g wet weight of cyanobacteria biomass in 100 ml methanol with occasional shaking for 24 hr at room temperature. The residual cells were separated by passing through Whatmann No11 filter paper and crude extracts were fractionated into hexane and ethyl acetate fractions. The organic fractions were concentrated to dryness using rotary evaporator at 40 °C, dissolved in DMSO to a final concentration of 1000 µgml^{-1} and stored at -20°C until used. The absorbance spectrum of the extracts from 300 to 700 nm was measured in a multimode microplate reader (Biotek instruments, USA).

Phytochemical analysis of extracts

Phytochemical analysis were carried out following standard protocols to qualitatively analyze the presence of various compounds such as alkaloids, carbohydrates, proteins, terpenoids, phenols, tannins, flavanoids, steroids and saponins in the extract (Antony *et al*., 2011). Here the indicator reagents were added to separate glass tubes containing 1 ml aliquots of extracts (100 µgml^{-1}), and the presence of phytochemicals were recorded as color change. Based on the intensity of color formation, the hexane and ethyl acetate fractions were classified into negative (-), low (+) and high (++) for the presence of different phytochemicals. The presence of alkaloids was confirmed with the formation of reddish-brown precipitate on addition of few drops of Wagner's reagent. Wagner's reagent was prepared by dissolving one gram of iodine and three grams of potassium iodide in 50 ml of distilled water. Carbohydrates were confirmed by the formation of brick-red precipitate on boiling the extract with equal volume of Fehling's reagents A and B for one minute. Proteins in the extract formed violet coloration on addition of equal volume of Ninhydrin solution (0.1% in methanol). Phenols and tannins formed dark green color on addition of few drops of 5 % $FeCl_3$ to the extract. Formation of reddish-brown precipitate upon addition of concentrated H_2SO_4 to a solution of the extract in chloroform confirmed presence of terpenoids. In the same assay, formation of greenish yellow in the lower H_2SO_4 layer and reddish brown ring in the upper layer indicated the presence of steroids. Flavanoids form yellow color on addition of few drops of NaOH (1N) solution, which becomes colorless upon acidification with HCl (1N). Formation of froth on addition of few drops of water, oil or Na_2HCO_3 to the extract confirmed presence of saponins.

Determination of antioxidant activity

Three different bioassays, *viz.*, ABTS [2, 2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid] decolouration assay, DPPH (1-diphenyl-2-picrylhydrazyl (α, α-diphenyl-β-picrylhydrazyl) radical scavenging assay and total antioxidant activity by FRAP (Ferric reducing antioxidant power) method were employed. In ABTS decolouration assay, the ABTS radical cations (ABTS*) were generated by treating ABTS stock solution (7mM) with 2.45 mM potassium persulfate at room temperature, in dark for 12–16 hr. Varying concentrations of hexane and ethyl acetate fractions (20, 40, 60, 80, 100 µgml^{-1}) were added separately to one ml of diluted solution containing ABTS* (A_{734} 0.706) and incubated at room temperature for seven minutes. Absorbance was measured at 734 nm using BHT as positive control. Negative control without any extract was also maintained. The percentage ABTS* scavenging activity was calculated using the formula:

$$\text{ABTS* Scavenging activity (\%)} = \left\{\frac{N1-N2}{N1}\right\} x100$$

Where N1 and N2 are the absorbance of control and sample groups.
In DPPH assay, different concentrations of the organic fractions (100-300 µgml^{-1}) were mixed with 1.0 ml of 0.135 mM DPPH in methanol, made up to a final volume of 2.0 ml and kept in dark for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid was used as positive control. The radical scavenging activity was determined based on percentage inhibition of absorbance, which was calculated using the following formula:

$$\text{Inhibition of absorbance (\%)} = \left\{\frac{N1-N2}{N1}\right\} x100$$

Where N_1 and N_2 are the absorbance of control and sample groups.
For FRAP assay different concentrations of the organic fractions (100-500 µg ml^{-1}) were allowed to react with 2.85 ml of substrate solution in a final volume of 3.0 ml for 30 min under dark. The substrate solution was prepared by mixing acetate buffer (300 mM, pH 3.6): TPTZ (2,4,6-tripyridyl-s-triazine 40 mM): $FeCl_3 \cdot 6H_2O$ (20 mM) in a ratio of 10:1:1. Absorbance of the coloured product (ferrous tripyridyltriazine complex) was measured at 593 nm. Ascorbic acid was used as a standard and activity values were calculated from slope of the standard curve. Results were expressed in µg ml^{-1} ascorbic acid.

Cytotoxicity assay

Cytotoxicity of the hexane fractions of *Leptolyngbya* sp and *Limnothrix* sp, having higher antioxidant activity, were estimated using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium chloride (MTT) assay. Here, ~1x10^6 mouse embryonic fibroblast cells (3T3) were inoculated into 96 well tissue culture plates containing Dulbecco's modified Eagle's medium [DMEM] supplemented with 10 % *fetal bovine serum* [FBS*]* and incubated for 48 hr at 37 ^{O}C. The cells were washed with phosphate-buffered saline [PBS], and the medium was exchanged with DMEM containing different sample concentrations (100, 200, 300, 400 and 500 µg ml^{-1}) of hexane fractions of cyanobacteria. The cells were incubated for 24 hr at 37 ^{O}C and subjected for MTT assay following standard protocol. Briefly, cells were washed and supplemented with 50 µl of MTT solution (5 mg ml^{-1}) prepared in PBS and kept for incubation under dark at 37 ^{O}C for 3 hr. Subsequently, the viability of the cells was measured as a function of reduction of MTT to insoluble formazan by mitochondrial dehydrogenase enzyme of healthy cells. Formazan crystals were dissolved in dimethyl sulphoxide and the absorbance was recorded at 570 nm using a multimode microplate reader (Biotek instruments, USA). All tests and analyses were run in triplicate.

UV treatment and ROS assay

The efficiency of the hexane fraction of *Limnothrix* sp in protecting 3T3 cells from UV induced ROS toxicity was measured using CellROx deep red oxidative stress detection reagent (Molecular probes, Life Technologies USA). Here, 3T3 cells prepared in 96 well microplate were supplemented with PBS (pH 7.4) containing 100 µg ml^{-1} hexane fraction and exposed to 200 Jm^{-2} UV light (254 nm UV-C) using a UV gene linker apparatus equipped with an energy output control. Control cells without extract under UV and without UV exposure were also maintained. Cells were washed copiously with PBS and stained with CellROX Deep red reagent following the protocol of the manufacturer. CellROX Deep red reagent is a fluorogenic probe designed to reliably measure ROS in live cells. CellROX Deep Red Reagent was added at a final concentration of 25 µM to the cells, and then incubated for 30 min at 37 ^{O}C. Subsequently, the cells were washed with PBS and the fluorescent intensities were measured at excitation/emission maxima at 640/665 nm using a multimode microplate reader (Biotek instruments USA).

RESULTS AND DISCUSSION

Globally, demand for non-cytotoxic antioxidant molecules as food preservatives and in health management are currently on the rise. Earlier studies have established antioxidant properties from crude extracts and fractions of several cyanobaceteria, *viz*., *Oscillatoria* sp, *Microcystis aeruginosa, Cylindrospermopsis raciborskii* **(Babu & Wu, 2008)**, *Synechococcus* sp, *Chlamydomonas nivalis* and *Nostoc ellipsosporum* **(Li *et al*., 2007)**. Chemical examination had indicated phenols and microsporine like aminoacids to be the prominent antioxidant metabolites of cynobacteria **(Takamatsu *et al*., 2003)**. Another interesting group of compounds are pycocyanins from *Oscillatoria tenuis* **(Thangam *et al*., 2013)**. Flavanoids, the prominent antioxidants in terrestrial plants have also been reported from marine cyanobacteria **(Booiji-James, Dube, Jansen, Edelman, & Mattoo, 2000; Rastogi *et al*., 2010; Wada, Sakamoto, & Matsugo, 2013)**. The phytochemical analysis showed that the hexane extract of the *Leptolyngbya* sp and *Limnothrix* sp were rich in flvanoids, steroids and terpenoids (Table 1). The UV-Vis absorption spectra of the hexane and ethyl acetate fractions (Figure 1) were comparable to that of flavanoids, the well known antioxidant molecules found in terrestrial plants and cyanobacterial sources **(Rastogi *et al*., 2010; Wada *et al*., 2013)**. Flavanoids are a class of low molecular weight natural polyphenoic compounds that have the ability to donate a hydrogen atom or an electron in order to form stable radical intermediates, which renders them antioxidant properties (Hajimahmoodi *et al*., 2010). Their chemical diversity, absorption properties and biotechnological potentials as antioxidant, antibacterial, antioncogenic and antiinflammatory agents are reviewed (Rastogi *et al*., 2010; Ververdis *et al*., 2007).
The antioxidant activity of hexane fractions of *Limnothrix* sp and *Leptolyngbya* sp were confirmed using ABTS decolouration assay, DPPH radical scavenging activity and total antioxidant activity by FRAP assay. The superoxide anion radical scavenging properties of the fractions were measured as its ability to decolorize ABTS*. The maximum percentage of decolourization was observed with hexane fraction of *Leptolyngbya* sp. (IC_{50}=30 µg ml^{-1}) while the other fractions showed comparable activity at relatively higher concentrations (IC_{50} 60-80 µg ml^{-1}, Figure 2a). The DPPH assay (Figure 2b) revealed a dose-dependent activity, with highest being shown by the hexane fraction of *Limnothrix* sp (IC_{50}=260 µg ml^{-1}), followed by the two ethyl acetate fractions, while the hexane fraction of *Leptolyngbya* sp had low activity (IC_{50}=330 µg ml^{-1}). FRAP assay revealed the reducing potential of these fractions. Here too, the hexane fractions of *Limnothrix* sp. was found the most promising (IC_{50}=117 µg ml^{-1}), followed by

hexane fraction of *Leptolyngbya* sp. (IC_{50}=176 µg ml^{-1}, Table 2). These values are promising and comparable to the antioxidant extracts from other terrestrial **(Antony *et al*., 2011; Boonchum *et al*., 2011; Rodrigues, Mariutti, & Mercadante, 2012; Sajeesh, Arunachalam, & Parimelazhagan, 2011)** and cyanobacterial sources **(Shanab, Mostafa, Shalaby, & Mahmoud, 2012)**. Previously, the antioxidant properties of the hexane extracts of *Anabaena* sp.,*Chrococcus* sp., *Nostoc* sp., *Microchaete* sp., *Tolypothrina* sp. and *Fischerella* sp were evaluated and found positive correlation with the level of phenolic compounds **(Hajimahmoodi *et al*., 2010)**. The aqueous extract of *Anabaena flous-aquae* and *Nostoc humifusum* reported the antioxidant properties ($IC_{50} \leq$ 100 µg ml^{-1}), while they were toxic at this concentrations **(Shanab *et al*., 2012)**. Interestingly, the non-cytotoxic fractions such as ethanol extracts of *Phellinus merrillii*, methanol extract of *Agelas oroides* and aqueous extract of *Saccharomyces cerevisiae* showed antioxidant property at very high concentrations **(Heng-yuan CHANG, 2007; Orhan *et al*., 2012; Phonnok, Tanechpongtamb, & Wongsatayanon, 2010)**. Although an ideal antioxidant molecule for application in food and health care products are exptected as noncytotoxic and active in cellular environment, many of the synthetic as well as natural antioxidants were reported to be cytotoxic too **(Kahl & Kappus, 1993; Shanab *et al*., 2012)**. *In vitro* assays indicate the ability of an antioxidant molecule to donate electrons to stabilize free radicals, but do not indicate its cytotoxicity or efficiency in scavenging intracellular ROS. It is possible that some antioxidant molecules, after donating electrons, might become toxic to host cells. Interestingly the hexane fraction of *Limnothrix* sp showed antioxidant properties without inducing any cytotoxicity. Here the cytotoxicity of hexane fractions to mouse fibroblas 3T3 cells were studied using standard MTT assay. In MTT assay, the viability of animal cells were measured as their ability to produce mitochondrial dehydrogenase enzyme which reduce yellow MTT into purple formazan. A dicrease in the reduction of MTT is an index of mitochondrial damage and cell death. During our studies, *Leptolyngbya* sp (IC_{50}: 200 µg ml^{-1}) was found more toxic than *Limnothrix* sp. (IC_{50}: 400 µg ml^{-1}) (Figure 3). Presence of alkaloids, as revealed in the phytochemical analysis of the hexane fraction of *Leptolyngbya* sp. could be the reason for its higher toxicity. Our result is also in agreement with earlier reports on toxicity of this organism **(Costa *et al*., 2014)**. Recent studies reported that roughly one third of terrestrial and half of marine cyanobacteria are potenitally exploitable for bioactive molecules with cytotoxic potential **(Hrouzek *et al*., 2016; Raja, Hemaiswarya, Ganesan, & Carvalho, 2015)**.

The ability of hexane fraction of *Limnothrix* sp to protect 3T3 cells from UV induced ROS stress was assayed using CellROX Deep red reagent (Molecular probes by Life Technologies, USA). Here, the cell permeable deep red reagents are non-fluorescent in reduced state, but exhibit strong fluorogenic signals upon oxidation by UV induced ROS. Exposure of 3T3 cells to UV radiation for 5 minutes increased ROS production by 1.6 times while it was comparable with control levels in cells supplemented with 100 µg ml^{-1} of the above hexane fraction of *Limnothrix* sp (Figure 4). Under normal conditions, the UV radiations induce direct or indirect toxicities to animal cells. High energy carrying UV-B photons (280 – 315 nm) cause skin reddening and sunburns, damage the superficial epidermis and plays a key role in the development of skin cancer. On the other side, UV-A radiation (315 – 400 nm) transfers energy to molecular oxygen ($^3O_2$), leading to the generation of singlet oxygen ($^1O_2$) and initiate a cascade of energy transfer reactions leading to the production of reactive oxygen species (ROS). A minimum concentration of ROS is essential for maintaining the regular metabolism of cells, and antioxidants are available in cells for self protection from ROS mediated toxicities. The elevated levels of ROS induce damage to DNA and protein leading the the development of extreme chronic conditions like ageing, arthritis, diabetes, tissue damage, muscular dystrophy and neurological disorders. Aslo, ROS are known to be the prominent cause of food decay, leading to rancidity, toxicity and destruction of biochemical components important in physiological metabolism. ROS stress assay results in live 3T3 cells indicates that the hexane fraction of *Limnothrix* sp can scavenge intracellular ROS and thus could be a potential antioxidant for application at cellular level.

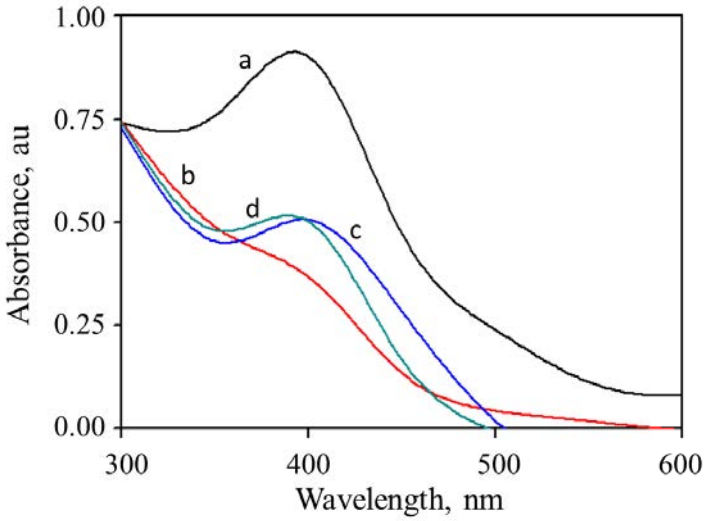

Figure 1 Absorption spectra of hexane (a,c) and ethyl acetate (b,d) fractions of *Limnothrix* sp (a,b) and *Leptolyngbya* sp (c,d)

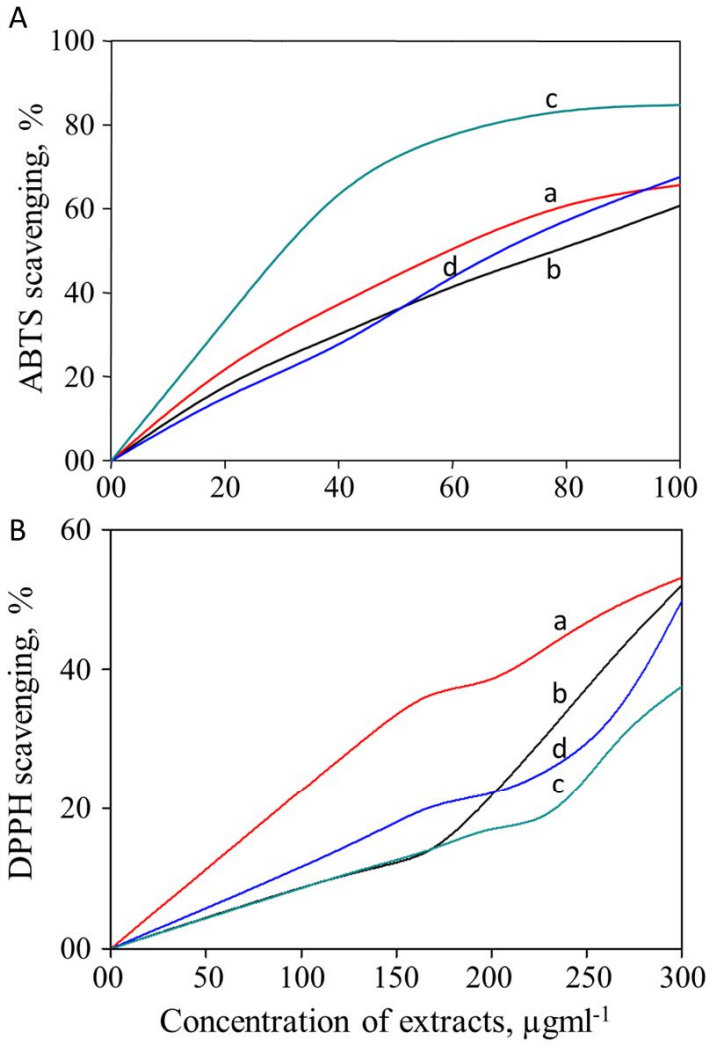

Figure 2 Antioxidant properties of hexane and ethyl acetate fractions of the samples expressed as their ability to scavenge ABTS (A) and DPPH (B) radicals. Hexane (a,c) and ethyl acetate (b,d) fractions of *Limnothrix* sp (a,b) and *Leptolyngbya* sp (c,d) are labelled

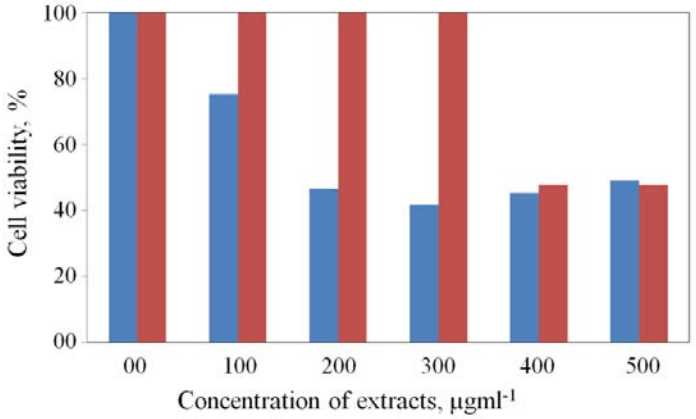

Figure 3 Viability of mouse fibroblast 3T3 cells in the presence of increasing concentrations of hexane extracts from *Limnothrix* sp (red) and *Leptolyngbya* sp (blue)

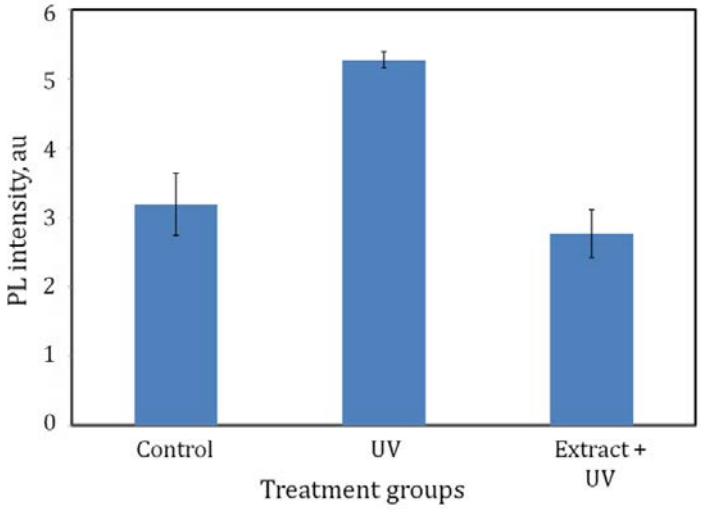

Figure 4 Efficiency of hexane extract (100 µg ml^{-1}) of *Limnothrix* sp in scavenging UV induced intracellular ROS measured by CellROX deep red reagent. The photoluminescence (PL) intensity indicated the mean concentration of ROS in 3T3 cells, without UV (control), with (UV) and in the presence of extract (extract +UV)

Table 1 Phytochemical analysis of hexane and ethyl acetate fractions of *Leptolyngbya* and *Limnothrix* sp. The results are expressed as negative (-), positive at low (+) and high (++) levels.

Phytochemical	***Leptolyngbya* sp**		***Limnothrix* sp**	
	Hexane	Ethyl acetate	Hexane	Ethyl acetate
Alkaloids	++	++	+	-
Carbohydrate	-	+	-	+
Aminoacids	-	+	-	+
Phenols & Tannins	-	+	-	+
Terpenoids	++	+	++	++
Saponins	-	-	-	-
Flavanoids	++	-	++	++
Steroids	++	+	++	++

Table 2 Antioxidant properties of hexane and ethyl acetate fractions of samples expressed as their ability to reduce ferric ions (FRAP assay)

	FRAP values (µg/Ascorbic acid equivalent)	
Extracts	***Leptolyngbya sp***	***Limnothrix* sp**
Hexane	176	117
Ethyl Acetate	260	225

CONCLUSION

Though cyanobacteria are known to be a rich source for antioxidants, toxicity prevents their widespread usage as antioxidants in food/cosmetics. We were particularly interested in identifying safe antioxidants which could be used directly, without any cumbersome de-toxification processes. To evaluate these contrasting properties, we selected two filamentous cyanobacteria ubiquitous in Arabian Sea and evaluated their toxicity and antioxidant property using specific assays. At the same time, a qualitative idea, regarding different types of compounds present in them were obtained through specific phytochemical analyses. The hexane fraction of *Limnothrix* sp was found to be rich in non-cytotoxic antioxidant molecules which are also capable of protecting animal cells from UV induced ROS stress. The above benevolent properties make this organism a good choice for non-toxic antioxidant products for use in beverages and other food products and in cosmetics. At the same time, the higher cytotoxicity of the hexane fraction of *Leptolyngbya* sp. may be useful in cancer therapy and is to be explored further.

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EFFECT OF ROSELLE CALYX EXTRACT ON GINGIPAIN ACTIVITY, PRODUCTION OF INFLAMMATORY CYTOKINES, AND ORAL BACTERIAL MORPHOLOGY

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ABSTRACT

This study to investigate the effect of roselle calyx extract on gingipain activity, production of inflammatory cytokines, and oral bacterial morphology. The inhibitory effect of roselle calyx extract (RCE) on Arg- (Rgp) and Lys-gingipain (Kgp) was evaluated using synthetic substrate colorimetric assay with a 96-well microtiter plate. RCE with various concentrations was tested for determination of interleukin (IL)-6 and IL-8 produced from KB cells stimulated with heat-inactivated *P. gingivalis.* The production of IL-6 and IL-8 was quantified using an ELISA kit. The morphological alterations in cells of *S. mutans* and *P. gingivalis* after treatment with RCE were studied using scanning electron microscopy (SEM). RCE could inhibit both Rgp (90%) and Kgp (70%) activities significantly at concentrations lower than the minimum inhibitory concentration (MIC) (2.2 mg/mL). Furthermore, the production of IL-6 and IL-8 from KB cells stimulated with *P. gingivalis* was significantly inhibited by RCE at 6 h after exposure, in a dose-dependent manner. After treatment with RCE, the morphological alterations were observed in cells of *S. mutans* and *P. gingivalis* by using SEM. The ability of RCE to inhibit gingipain activity and production of inflammatory cytokines indicates that RCE could be considered for prevention and clinical treatment for periodontitis.

Keywords: Roselle calyx extract, Rgp, Kgp, *Porphyromonas gingivalis*, IL-6, IL-8

INTRODUCTION

Periodontitis is an oral infectious disease caused by bacteria or groups of bacteria. The bacteria accumulate on the surface of the tooth and cause the destruction of periodontal connective tissue and alveolar bone, resulting in tooth loss (**Haake *et al*., 2006; Darveau, 2010**). It was reported that a group of bacteria, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans*, *Treponema denticola,* and *Fusobacterium nucleatum* may contribute to periodontal diseases. Among these, *Porphyromonas gingivalis* (*P. gingivalis*), a Gram-negative, anaerobic, non-motile rod, is strongly associated with adult periodontitis (**Slots *et al*., 1994**). This species is significantly more abundant in diseased sites than in healthy sites (**Slots *et al*., 1999; Haffajee *et al*.,1994**). The presence of *P. gingivalis* is indicative of disease progression risk and reduced levels correlates with successful clinical treatment (**Haffajee *et al*.,1994**).

P. gingivalis possesses many virulence factors, including gingipains. Gingipains are trypsin-like cysteine proteinases classified into two groups, the arginine-specific cysteine protease (Rgp) and the lysine-specific cysteine protease (Kgp), based on substrate specificity. Rgp activates the blood coagulation system, associated with gingival crevicular fluid production, and initiates the inflammation process (**Imamura, 2003**). Kgp contributes to a tendency toward gingival bleeding (**Imamura, 2003; Potempa *et al.*, 2000**). The gingipains have been isolated from culture supernatants, vesicle membrane fractions, and cell extracts (**Chen *et al*., 2001; Yamanaka *et al*., 2007**).

Epithelial cells, which line mucosal surfaces and provide an important mechanical barrier, play a communication role for the host as microorganism sensors and signal providers. These cells can activate mucosal inflammatory and immune responses(**Kagnoff *et al*., 1997**). It has been reported that *P. gingivalis* can bind to and invade human oral epithelial cells (**Duncan *et al*., 1993**). An interaction between bacteria and epithelial cells is required for periodontal inflammation (**Yumoto *et al*., 1999**). Cytokines, small soluble proteins produced by a cell, play an important role in many biological activities such as inflammation (**Okada *et al*., 1998**). Inflammatory cytokines, like interleukin-(IL) 6 and IL-8, are induced during the inflammatory process. Some reports showed the involvement of IL-6 and IL-8 in the pathogenesis of tissue destruction in periodontitis (**Yumoto *et al*., 1999; Okada *et al*., 1998; Kang *et al*., 2011**). Since gingipains, IL-6, and IL-8 are involved in the pathogenesis of periodontitis, these components could be effective targets for prevention/control of periodontitis.

Roselle calyx extract (RCE), the extract from *Hibiscus sabdariffa* L. (Family Malvaceae) grows in tropic and subtropic areas. Roselle calyx has medicinal properties and has been reported to contain alkaloids, saponins, and flavonoids such as gossypetine, hibiscetin, and sabdaretine (**Hirunpanich *et al*., 2005; Olaleye *et al*., 2007**). It also contains hibiscus acid, hydroxybenzoic acids, flavonols, anthocyanins, and other polyphenolic compounds (**Rodrigues *et al*., 2011**). RCE has been considered to have antihypertensive (**Arellano *et al*., 2004**) hepatoprotective (**Ali *et al*., 2003**), antihyperlipidemic (**Hirunpanich *et al*., 2006**), antioxidant (**Yang *et al*., 2012**), anticancer (**Tsai *et al*., 2014**), and antimicrobial (**Olaleye *et al*., 2007; Nwaiwu *et al*., 2011**) properties. It is possible that the use of plant extracts with the ability to inhibit gingipains activity and cytokine production is an alternative strategy for periodontal therapy. Our previous study showed that RCE has antibacterial activity and inhibits biofilm formation. In the present study, we investigate RCE's ability to inhibit gingipains activities and cytokine production stimulated by *P. gingivalis*, and its effect on bacterial morphology.

MATERIAL AND METHODS

Preparation of the RCE

We soaked 16 g powder of dried roselle calyx in 160 mL ethyl alcohol (Wako Pure Chemical Industries Ltd) with shaking for 24 h at room temperature. After centrifugation, the extract was lyophilized. Then the extract was dissolved in phosphate-buffered saline (PBS) and the pH was adjusted to 7.0. The extract was aseptically filtered through a disposable membrane filter unit with a 0.45-µm pore size. The extract was stored at - 20°C for further use.

Determination of Rgp and Kgp activity

The inhibitory effect on Rgp and Kgp of RCE at different concentrations was evaluated by using synthetic substrate colorimetric assay (**Yamanaka *et al*., 2007; Nakatsuka *et al*., 2014**). Benzoyl-arginine-*p*-nitroanilide (Sigma-Aldrich) and tosyl-glycine-proline-lysine-*p*-nitroanilide (Sigma-Aldrich), in 100 µl of 0.1 M Tris-HCl (pH 8.0) containing 1 mM dithiothreitol were used as substrates (final concentration 0.5 mM) for Rgp and Kgp, respectively. The substrates were dispensed into the wells of a 96-well microtiter plate. A bacterial cell suspension (50 µl) of *P. gingivalis*, 1 x 10^8 cells/ml in PBS and RCEs at different concentrations, were added to the substrate and incubated at 37°C for 50 min. The optical density, OD, of each well was measured by microtiter plate reader at a wavelength of 405 nm (A_{405}). Relative enzymatic activity was determined as follows: [(A_{405} with bacterial cells and RCE − A_{405} of control) / (A_{405} with bacterial cells − A_{405} of control)] x 100. Degradation obtained in the absence of the RCE was given a value of 100%.

Determination of IL-6 and IL-8 production

KB cells (a human mouth epithelial cell line) were grown in a 5% CO_2 incubator at 37°C in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (100 µg/ml). After washing twice with PBS, the cells were detached from the cultured plate and then diluted to obtain 1 x 10^5/ml. A cell suspension (100 µl) was cultured in a 96-well plate and incubated to about 90% - 100% confluence. Bacterial cells of *P. gingivalis* (1 x 10^8 cfu) were suspended in serum-free DMEM and heat-inactivated at 80°C for 30 min before infection of KB cells. RCE in various concentrations and 60 µl of the bacterial suspension were incubated with KB cells at a multiplicity of infection (MOI) of 200 at 37°C in 5% CO_2. After 6 h, the supernatants were collected and stored at -20°C for cytokine assays. The levels of cytokine in the culture supernatant were determined by ELISA kit (Thermo, USA) according to the manufacturer's instructions. Briefly, the lyophilized standard or RCE was placed on a 96-well strip plate precoated with anti-human cytokine. Afterward, biotinylated antibody reagent was added to each well, followed by streptavidin-horseradish peroxidase solution. Tetramethylbenzidine (TMB) substrate was added and the reaction was stopped by adding stop solution containing 0.16 M sulfuric acid. The absorbance was measured on a plate reader at 450 nm.

Examination of bacterial morphology with Scanning Electron Microscopy

A scanning electron microscopy (SEM) was performed to examine the changes of bacterial morphology after treatment with RCE. In this study, *S. mutans* ingbritt and *P. gingivalis* ATCC 33277^T were used. Bacterial suspensions were adjusted to reach OD 1.0 at 600 nm in PBS after overnight culture and washing. These suspensions were layered on cell culture coverslip disks (Thermo Scientific, Rochester, NY, USA) by soaking for 2 h in anaerobic conditions. The layered disks were washed three times and then treated either with RCE at three times minimum inhibitory concentration (MIC) (21.6 mg/mL) or with PBS for 1 h or 2 h at room temperature. After being washed, they were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 24 h. The resultant glutaraldehyde-treated layered disks were then washed and dehydrated in ethanol (50, 70, 80, and 90% successively), each for 10 min, and finally three times with 100% ethanol for 20 min each. They were dried by the CO_2 critical-point drying technique (HCP-2, Hitachi, Tokyo, Japan), coated with gold in vacuum (Eiko, IB-3 ion coater, Japan) and examined using SEM (Hitachi S-3500N, Japan).

Statistical analysis

Statistical analysis was performed using SPSS 21 software. Results were obtained in triplicate and were expressed as mean ± standard deviation (SD). The significance of the differences between groups was determined using independent *t*-test with a value of $p< 0.05–0.01$; the effect of RCE on *P. gingivalis*-induced IL-8 expression in KB cells was determined using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test.

RESULTS

Effect on Rgp and Kgp activity

The effect of RCE on Rgp and Kgp activity was evaluated by colorimetric assay using synthetic substrates (Figure 1). The present study showed that RCE exhibited inhibitory effect on both Rgp and Kgp activity in a dose-dependent manner. RCE reduced the activity of Rgp about 60% at 0.6 mg/mL, 80% at 1.1 mg/mL, and 90% at 2.2 mg/mL. The reduction in Kgp activity was about 50% at 0.6 mg/mL, 60% at 1.1 mg/mL, and 70% at 2.2 mg/mL. The differences in gingipains activity between the untreated and treated groups were statistically significant ($p<0.05$).

Effect on IL-6 and IL-8 production

We evaluated the effect of RCE at various concentrations on the production of IL-6 and IL-8 by KB cells stimulated with the heat-inactivated *P. gingivalis*. In Figure 2, the levels of IL-6 (Figure 2A) and IL-8 (Figure 2B) produced by KB cells were significantly reduced by treatment with RCE in a dose-dependent manner ($p < 0.01$) with similar reduction patterns.

Effect on bacterial morphology

Figure 3 shows the morphological alteration of *S. mutans* and *P. gingivalis* after treatment with RCE for 1 h (3E and G) and 2 h (3F and H) compared to the structure of the controls (3A, B, C, and D). Bacterial cells of *S. mutans* and *P. gingivalis* in the control group had regular shapes and smooth surfaces. After treatment with RCE, some *S. mutans* cells showed irregular changes such as enlargement and clumping of cells. In *P. gingivalis*, treatment with RCE caused aggregation and distortion of cells. These morphological alterations increased after 2-h treatment.

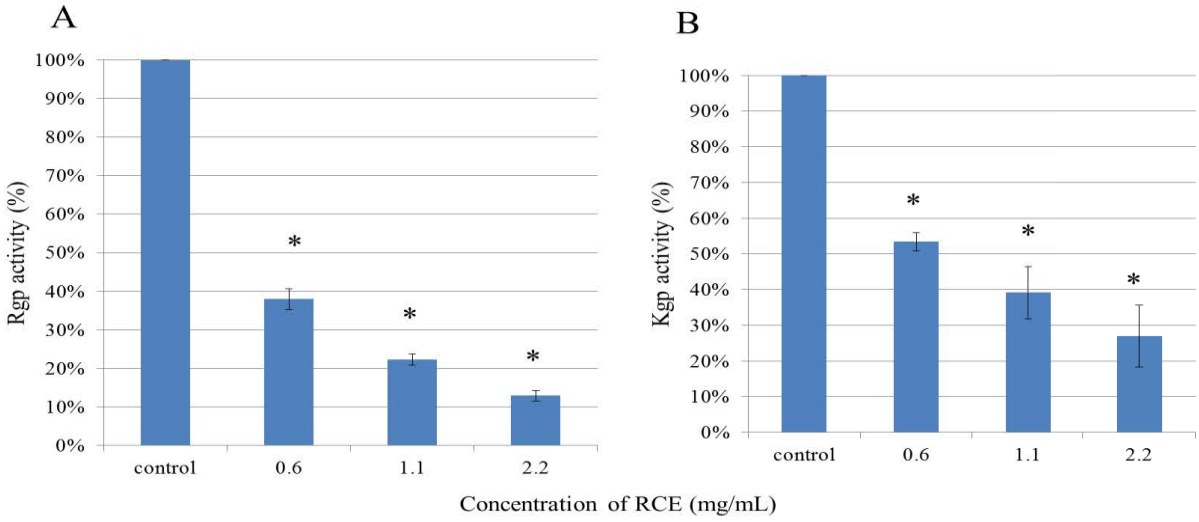

Figure 1 Effect of roselle calyx extract on Rgp (A) and Kgp (B) activity of *P. gingivalis*. The data was expressed with the mean ± SD in triplicate experiment. * $p < 0.05$: significantly different from the control. Control: absence of RCE; Rgp: Arg-gingipain; Kgp: Lys-gingipain; RCE: roselle calyx extract.

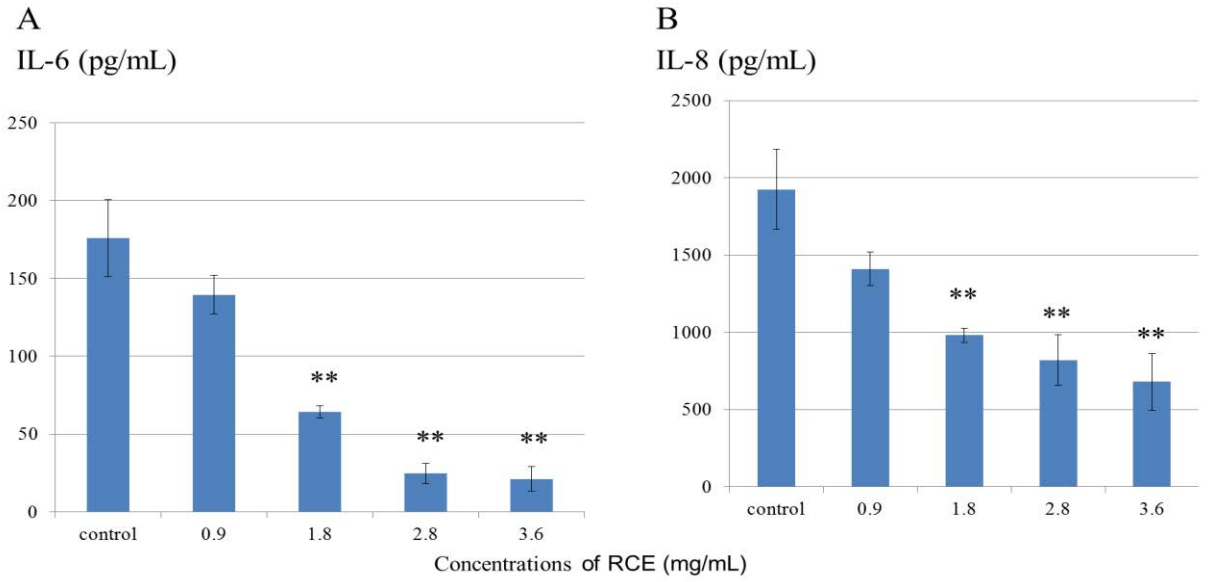

Figure 2 Effect of RCE on *P. gingivalis*-induced IL-6 (A) and IL-8 (B) expression in KB cells. Control: PBS, *P. gingivalis* was used at MOI 200. ** $p \leq 0.01$: significantly different from the control. The data is expressed with the mean ± SD in triplicate experiment.

DISCUSSION

Many bacterial species have been identified in periodontal pockets of patients; among those *P. gingivalis* is frequently isolated from the lesions of chronic periodontitis. It was reported that *P. gingivalis* produced major proteinases, Arg-specific gingipain (Rgp) and Lys-specific gingipain (Kgp), as virulence factors (**Kataoka *et al*., 2014**). Gingipains play a key role in the pathogenic function of *P. gingivalis*. They have the ability to degrade a number of host proteins significant for bacterial growth and metabolism, and are responsible for dysregulation of host defensive inflammatory reaction and failure of the host to eliminate bacteria (**Olsen *et al*., 2014**).

Gingipains are located on cell surfaces and in culture supernatants, and are related to extracellular vesicles (**Chen *et al*., 2001**). The gingipain-loaded outer membrane vesicles may contribute to tissue destruction in periodontal diseases by serving as a vehicle for the antigens and active proteases (**Nakao *et al*., 2014**). When separate proteinases with arginine and lysine specificity were isolated from a high molecular mass fraction of the *P. gingivalis* culture fluid, the arginine- and lysine-gingipains were found to contain a hemagglutinin complex. This hemagglutinin may be important in the uptake of hemin via hemagglutination and subsequent hemolysis of erythrocytes (**Pike *et al*., 1994**). Arginine-specific cysteine proteinase from the culture supernatant of *P. gingivalis* has the ability to disrupt the functions of polymorphonuclear leukocytes. Further, the enzyme was suggested as a major virulence factor from *P. gingivalis* in the progression of periodontal diseases due to direct destruction of periodontal tissue components and the disruption of the normal host defense mechanism (**Kadowaki *et al*., 1994**). The culture supernatant from *P. gingivalis* also induces the disruption of the adhesion and proliferation activities in the endothelial cells; Rgp and Kgp are responsible for these activities (**Baba *et al*., 2002**). It is clear that while gingipains are important for the survival of bacteria, they are also pathogenic to the host. Therefore, both Rgp and Kgp activities should be potential therapeutic targets (**Olsen *et al*., 2014**).

Results in our study indicate that RCE inhibited the activity of Rgp and Kgp at a sub-MIC concentration (Figure 1). Our previous study reported that the MIC of *P. gingivalis* is 7.2 mg/mL (**Sulistyani *et al*., 2016**). The concentration of RCE used in this study was sub-MIC, therefore the decrease of Rgp and Kgp activity is not because of inhibition of bacterial growth, but because of inactivity of Rgp and Kgp. It was reported that RCE contains polyphenolic compounds (**Rodrigues *et al*., 2011**). Previous study showed that cranberry polyphenol was found to inhibit Arg- and Lys-gingipain activities in a dose-dependent manner (**Yamanaka *et al*., 2007**). Polyphenols from *Myrothamnus flabellifolia* Welw (MF) exhibit inhibition of *P. gingivalis* adhesion to epithelial cells, with strong influence against Arg-gingipain. In *P. gingivalis*, the gingipain proteases are suitable targets for polyphenol compounds. Proanthocyanidins react with bacterial surface proteins, leading to an unspecific cross-linking or denaturing effects of adhesion proteins and resulting in inhibition of Rgp (**Lőhr *et al*., 2011**). From these data, we assume that the inhibition effect of RCE on Rgp and Kgp may be due to polyphenol compounds. However, MF exhibits high toxicity, which may limit its clinical use, whereas RCE is safe to use.

Periodontopathogens can promote host inflammatory response by secretion of inflammatory mediators. The association of inflammatory mediators and local tissue destruction in periodontitis has been reported (**Okada *et al*., 1998; Birkedal, 1993; Bodet *et al*., 2007**). IL-6 is a pro-inflammatory cytokine that has been related to the pathogenesis of periodontal disease. IL-6 expression is increased at sites of periodontal inflammation. It has been suggested as a marker for periodontal disease activity (**Irwin *et al*., 1998**) and is involved in bone resorption (**Nakashima *et al*., 2000**). It was reported that IL-8, expressed in epithelial cells and macrophages, was observed in gingival inflammation. IL-8 may play an important role in the pathogenesis of periodontitis due to its pro-inflammatory and neutrophil chemotactic properties (**Okada *et al*., 1998; Kim *et al*., 2006**). The neutrophils are the first line of defense against periodontopathic bacteria. Continuous and excessive IL-8-mediated chemotactic and activation effects on neutrophils may contribute to local destruction of periodontal tissues (**Okada *et al*., 1998**).

In the present study, we analyzed whether KB cells stimulated by the heat-inactivated *P. gingivalis* for 6 h could produce IL-6 and IL-8. Previous study also reported that KB cells showed cytokine responses after infection with *P. gingivalis*, strongly comparable to that induced by *E. coli* in vitro (**Sandros *et al*., 2000**). The ability of epithelial cells to provoke the cytokine response was related to their adhesive and invasive capacity. In a comparative experiment using a primary culture of pocket epithelium and KB cells, the result revealed that cytokine responses after *P. gingivalis* and *E. coli* infection were similar.[36] In addition, in vivo study showed that *P. gingivalis* was more potent than *A. actinomycetemcomitans* in inducing proinflammatory cytokines expression. Animals infected with *S. gordonii*, considered a non-pathogenic bacteria, also induced IL-1ß and TNFα but not IL-6 (**Kesavalu *et al*., 2002**).

Our study exhibited that the production of IL-8 (1926 pg/mL) is higher than that of IL-6 (176 pg/mL). Our result is in accordance with previous studies; **Yumoto *et al*. (1999)** reported that the level of IL-8 production (approx. 2300 pg/mL) by KB cells stimulated with *E. corrodens* for 8 h is higher than the level of IL-6 production (approx. 98 pg/mL). The level of IL-6 increased 0.5 h after stimulation; the concentration increased constantly over time and it increased significantly within 4 h. In contrast, the level of IL-8 increased 4 h after stimulation, and increased aggressively from that point. The production of IL-8, higher than that of IL-6, was also shown in KB cells infected by *F. Nucleatum* (**Kang *et al*., 2011**).

The level of IL-6 and IL-8 response in KB cells induced by heat-inactivated *P. gingivalis* was reduced after treatment with RCE at all concentrations used in this study. No cytotoxic effects were detected by WST-1 assay in KB cells treated by RCE in those concentrations for 6 h (data not shown). It is indicated that the reduction of IL-6 and IL-8 production in KB cells treated by RCE is not associated with cell toxicity. In addition, treatment of uninfected KB cells with RCE did not affect cytokine responses (data not shown). It is shown that treatment by RCE does not affect KB cell production of IL-6 and IL-8.

It was reported that RCE contains polyphenols, with flavonoids being the largest of the dietary phenolics group (**Kim *et al*., 2006; Carretero *et al*., 2008; Medina *et al*., 2009**). Flavonoids have anti-inflammatory effects. Previous study showed that the non-dialyzable material (NDM) cranberry fraction, rich in polyphenolic compounds, could inhibit inflammatory mediator production (**Bodet *et al*., 2007**). The mechanism of inhibition may be via a down-regulation of the activator protein-1 (AP-1) activity. AP-1 and nuclear factor-κB (NF- κB) are known pro-inflammatory transcription factors that induce the production of cytokines and other pro-inflammatory molecules (**Verri *et al*., 2012**). AP-1 and NF- κB co-operatively regulate the synthesis of IL-6, IL-8, and PGE_2 in the gingival

connective tissue of patients with periodontitis (**Bodet *et al*., 2007; Kida *et al*., 2005**). Epigallocatechin gallate (EGCG), the major polyphenol component of green tea, inhibits IL-1ß-induced IL-8 production of nasal fibroblast and A549 epithelial cells. The activity might be associated with an intervening reactive oxygen species (ROS) pathway (**Kim *et al*., 2006**). Because the main compound of RCE is a flavonoid, we suggested that the flavonoid is involved in the inhibitory effect on IL-6 and IL-8 in KB cells induced by heat-inactivated *P. gingivalis*. The use of flavonoids as an anti-inflammatory may represent a better pharmacological approach compared to current therapies because flavonoids do not act by a single mechanism and present a better side effect profile, reducing such effects as gastrointestinal and renal lesions (**Kida *et al*., 2005**).

The morphologic alteration of oral bacterial species by treatment with RCE has not been reported previously. In this study, we investigated the effect of RCE on *S. mutans* and *P. gingivalis* using SEM. The layered disks of bacteria were treated with RCE at three times MIC for 1 h or 2 h under appropriate conditions. We used RCE at this concentration because our purpose was to observe morphologic alterations on the bacteria rather than a reduction in bacteria. In addition, using a concentration of RCE equal to MBC may have resulted in bacteria detaching from coverslip disks when dead bacteria were processed and removed. The SEM images illustrate the detrimental effects of RCE on the bacteria. *S. mutans* and *P. gingivalis* in the control group had regular shape and smooth surfaces. After 1 h of treatment, some cells of *S. mutans* showed irregular shape or clumping. After 2 h, some bacteria showed variation in size, some cells were expansive, and the clumping of cells was more evident than at 1 h of treatment. Treatment by RCE on *P. gingivalis* for 1 h caused aggregation and irregular cell outlines. These patterns were increased on cells treated by RCE for 2 h.

It has been reported that RCE contains phenolic compounds, such as flavonoid. The target site of flavonoid might be on membrane cell walls (**Cowan, 1999**). Our SEM result was in accordance with previous study and showed catechin, the polyphenol compound, caused leakage of intramembranous materials, strong aggregation, and bacterial cell pointing to the likely possibility of membrane fusion. It is well established that membrane fusion by polyethylene glycol, calcium ion, or virions has consistently resulted in the leakage of intramembranous materials and aggregation (**Ikigai *et al*., 1993**). SEM analysis of oral microorganisms treated with tea polyphenol showed major structural cell surface changes and irregular forms, with aggregates among cells (**Cho *et al*., 2010**). It was reported that damage to bacterial cells might manifest in a few ways, one of which was loss of membrane integrity resulting in leakage of essential intracellular constituents such as inorganic phosphate and proteins. The target of phenols was membrane integrity and leakage (**Denyer *et al*., 1998**). From the SEM observations, it seems that RCE binds to the cell membrane, penetrates into the phospholipid bilayer, and disrupts the membrane integrity, resulting in membrane leakage and cell aggregation. We suggested after aggregation, the bacteria will lose integrity and disintegrate, resulting in death. However, the mechanisms of RCE on oral bacteria remains unclear, therefore experiments need to be developed.

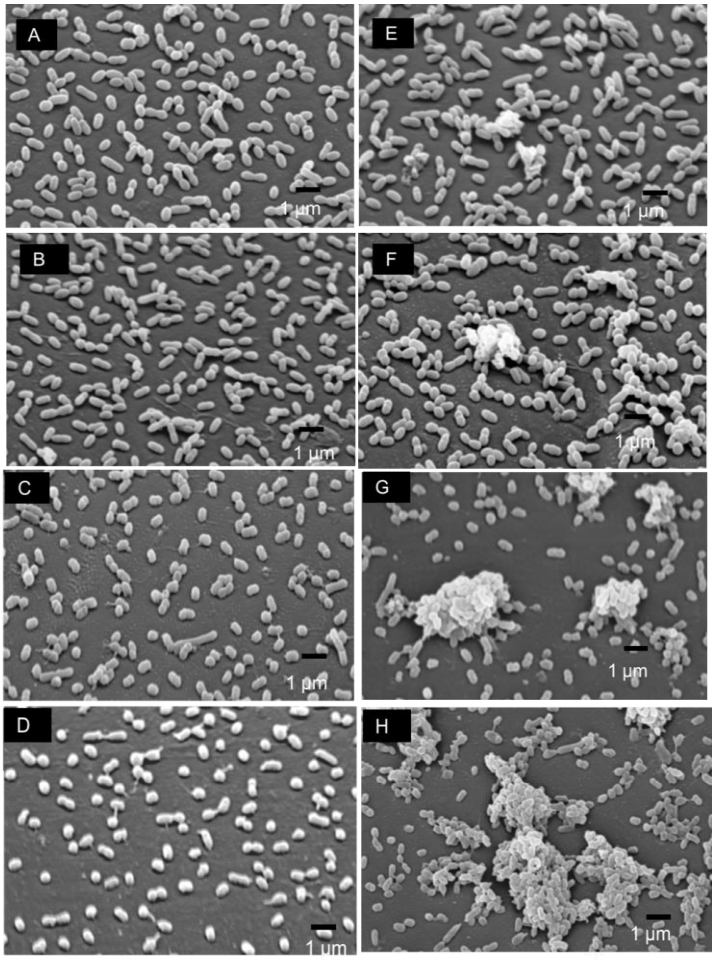

Figure 3 Scanning electron microscope *S. mutans* (A, B, E and F) and *P. gingivalis* (C, D, G and H). A and C: PBS 1 h; B and D: PBS 2 h; E and G: treatment with RCE for 1 h; D and H: treatment with RCE for 2 h.

CONCLUSION

Based on our results, RCE could inhibit the activity of Rgp and Kgp, the major proteases of *P. gingivalis*. Moreover, RCE inhibited IL-6 and IL-8 production in KB cells stimulated with the heat-inactivated *P. gingivalis*. From the SEM observations, we suggested that RCE binds to the cell membrane, resulting in membrane leakage and cell aggregation. These data suggest that RCE might have beneficial effect on prevention and clinical treatment for periodontitis.

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THE ANTIMICROBIAL AND ANTIOXIDANT POTENCIES OF *SATUREJA KHUZISTANICA* ESSENTIAL OIL FOR PRESERVING OF VEGETABLE OILS

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ABSTRACT

In recent years, the use of natural preservatives for protection of vegetable oils against microbial and chemical deterioration is one of the interesting issues. The purpose of this study was to evaluate the preservative activity of *Satureja khuzistanica* essential oil (SKEO) against microbial and chemical deterioration in sesame and flaxseed vegetable oils. Chemical composition of SKEO, chemical profiles, antioxidant and preservative potencies of inoculated vegetable oils with different concentration of SKEO were determined. Carvacrol was the main component of SKEO. The chemical profile of vegetable oils in presence of SKEO had no changes. Sesame and flaxseed vegetable oils had the IC_{50} equal to 26 and 22 µg/ml, respectively. Inoculation the SKEO (1%v/v) in vegetable oils decreased the IC_{50} for vegetable oils. SKEO showed promised antimicrobial activity against food microorganisms. Inoculation the SKEO (0.75%v/v) in sesame oil inhibited completely the bacteria and fungi after 14 days. Flaxseed oil inoculated with SKEO (1% v/v and lower concentrations) decreased the bacteria and fungi populations after 28 days. Therefore, the use of SKEO as natural preservative can protect vegetable oils from deterioration; also it gives the vegetable oils the other pharmacological effects such as anti-inflammatory and analgesic effects with applications in different industries.

Keywords: Preservative, Essential oil, *Satureja khuzistanica*, Antioxidant, Vegetable oil

INTRODUCTION

Inadequate drying process of oilseeds or poor situation in extracting the vegetable oils from oilseeds usually leads to microbial contamination in final products **(Okpokwasili and Molokwu, 1996)**. Microbial contaminations can make considerable changes in vegetable oils and finally affect on quality of these vegetable oils.
In contrast, the difference in intrinsic properties of vegetable oils can change the microbial load of vegetable oils. The fatty acid profile, phenolic compounds, tocopherol and sterol contents of vegetable oils have critical role in their intrinsic properties **(Gromadzka and Wardencki, 2011)**.
In addition to microbial contaminations in vegetable oils, the oxidants can affect on their shelf life **(Aluyor and Ori-Jesu, 2008)**. Benzoic acid, nitrites, sulfites as antimicrobial agents and butylated hydroxyltoluene (BHT), butylated hydroxyanisole (BHA), tocopherols and ascorbic acid as antioxidants are used for prevention the vegetable oils from spoilage. The application of chemical antioxidants and preservatives are associated with major health hazardous problems and toxicity **(Parke and Lewis, 1992)**. Furthermore, replacement the chemical and synthetic ones with natural agents in vegetable oils has great importance for many consumers of food, pharmaceutical and cosmetic industries in different parts of the worlds.
In these years, the use of essential oils as natural preservatives and antioxidants has increased as a new approach to overcome on these adverse effects **(Davidson and Taylor., 2007; Lambert *et al.*, 2001; Mahboubi *et al.*, 2014; Smith-Palmer *et al.*, 2001)**.
Satureja khuzistanica essential oil (Lamiaceae family) has been known for its antiseptic effects in traditional medicines. The antimicrobial activities of *S. khuzistanica* essential oil have been determined against a large number of bacteria and fungi *in vitro* conditions **(Akbari-Shahabi *et al.*, 2014; Sadeghi-Nejad *et al.*, 2010; Zarrin *et al.*, 2010)**. Other biological effects of *S. khuzistanica* such as anti-inflammatory **(Ghazanfari *et al.*, 2006)**, antinociceptive and analgesic **(Saberi *et al.*, 2013)** activities have been confirmed. Therefore, inoculation of *S. khuzistanica* essential oil into vegetable oils may be proposed it as suitable candidate for different industries especially in aromatherapy related therapies.
So, the aim of this study was to evaluate the preservative and antioxidant potencies of *S. khuzistanica* essential oil in sesame and flaxseed oils. These vegetable oils are two popular vegetable oils in different industries. Due to a positive relation between the chemical composition of essential oil and biological activities, we analyzed the chemical composition of *S. khuzistanica* essential oil and fatty acid profiles of vegetable oils in presence of this essential oil as natural preservative.

MATERIAL AND METHODS

S. khuzistanica essential oil and analysis of its chemical composition by Gas Chromatography (GC) and Gas chromatography–mass spectrometry (GC-MS)

Satureja khuzistanica essential oil with pale yellow color had been dedicated by Barij Essence Pharmaceutical Company of Iran. The chemical composition of essential oil were conducted on coupled Agilent technology (HP) 6890 with capillary column of HP-1MS (30 m × 0.25 mm, film thickness 0.25 µm) and Agilent technology (HP) 6890 with 5973 network mass selective detector system using GC and GC-MS apparatuses. The oven temperature program was initiated at 40 °C, held for 1 min then raised up to 230 °C at a rate of 3 °C /min, held for 10 min. Helium as the carrier gas at a flow rate of 1.0 ml/min with a split ratio equal to 1/50 injector were used. The detector and injector temperatures were 250 and 230 °C, respectively. Components of essential oil were identified by comparison with Retention indices (RI) relative to homologous series of n-alkanes (injected in conditions equal to samples ones) and by computer search using libraries of Wiley 275.L and Wiley 7n.1, as well as comparison of the fragmentation pattern of the mass spectra with data published in the literature **(Adams, 2001)**.

Microbial strains and the antimicrobial activity evaluation of essential oil

The microbial strains were including: *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 16404. The inhibition zones (IZ) diameters, the minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) values of essential oil were evaluated by disc diffusion and micro broth dilution assay as it reported elsewhere **(Mahboubi *et al*., 2014)**.

Table 1 GC and GC-MS analysis of *Satureja khuzistanica* oil

compound	RI	%
α-terpinene	929	0.2
p-cymene	938	0.5
β-phellandrene	941	0.1
γ-terpinene	971	0. 4
α-terpinolene	999	0.1
linalool	1013	0.1
carvacrol methyl ether	1142	0.1
thymol	1200	1.0
Carvacrol	1257	**84**
Eugenol	1295	0.2
carvacryl acetate	1303	0.02
trans-caryophyllene	1327	0. 4
α-bergamotene	1336	0.1
neryl acetone	1350	0.1
β-Bisabolene	1387	2.7

RI-retention index

Vegetable oils

Two samples of vegetable oils (sesame oil, flaxseed oil) were used. Vegetable oils were extracted from the seeds of *Sesamum indicum* (sesame oil) and *Linum usitatitissimum* (flaxseed) by cold press procedures in Glkaran Company, Kashan, Iran. The chemical properties of vegetable oils were determined as below. 0.2 ml of 1 M potassium hydroxide and 10 ml of methanol were added to 300 mg of each vegetable oil, then, it was refluxed for 10 min at 90 ºC. After cooling of this solution, the components of vegetable oils were extracted by hexane. 1 µl of extracted components was injected to GC-CP3800 (Varian) with a column of CP-Wax 52CB (50 m × 0.32 mm, film thickness 0.2 µm). Nitrogen was used as carrier gas (pressure 7 psi, flow rate 1.0 ml/min). The temperatures of detector and injector were 250 °C with split ratio of 10. The oven temperature program was initiated at 170 °C, held for 10 min, and then raised up to 250 °C with a rate of 3 °C /min. After that, it was held for 30 min at 250 °C.

Vegetable oils were inoculated by different concentrations of *S. Khuzestanica* essential oil (0.25, 0.5, 0.75, and 1% v/v). Vegetable oils alone were used as negative controls. Carvacrol as *S. Khuzestanica* essential oil indicator was detected in inoculated vegetable oil.
For this purpose, 1.5 g of each vegetable oil was mixed with 4.5 g ethanol (90 ºC) and 0.5 ml of hexanol (10 µg/ml). The mixture was put in water bath (60 ºC) for 5 min and then was put in cold water bath (4 ºC) for 10 min. Then, the mixture was centrifuged. 1 µl of supernatant was injected to GC-Sil 8CB (Varian) with column: CP-Wax52CB (60 m × 0.32 mm, film thickness 0.45 µm), nitrogen was used as the carrier gas (pressure 7 psi, flow rate of 1.0 ml/min, split ratio of 10). The temperatures of injector and detector were 230 and 250 °C, respectively. The oven temperature program was initiated at 50 °C, held for 10 min, then it was raised up to 230 °C at a rate of 3 °C /min and then was held for 70 min **(Pharmacopoeia, 2015a, b)**.

Table 2 Chemical profiles of vegetable oils and inoculated *S. khuzistanica* essential oil

Sample	IC_{50}	Essential oil	Vegetable oil (w/w %)				
	µg	carvacrol	Palmetic acid	Estearic acid	Oleic acid	Linoleic acid	α- linoleic acid
S	26	0.00	9.4	5.6	44.1	38.7	0.4
SS (0.25%)	21	0.21	9.4	5.6	44.1	38.7	0.4
SS (0.5%)	17	0.42	9.4	5.6	44.1	38.7	0.4
SS (0.75%)	14	0.58	9.4	5.6	44.1	38.7	0.4
SS (1%)	12	0.77	9.4	5.6	44.1	38.7	0.4
F	22	0.02	5.5	5.1	19.7	13.5	54.9
FS (0.25%)	14	0.20	5.5	5.1	19.7	13.5	54.9
FS (0.5%)	13	0.36	5.5	5.1	19.7	13.5	54.9
FS (0.75%)	10	0.56	5.5	5.1	19.7	13.5	54.9
FS (1%)	7	0.7	5.5	5.1	19.7	13.5	54.9

SS- Sesame oil+*S.khuzestanica* oil, FS- Flaxseed oil+ *S.khuzestanica* oil

Table 3 The antimicrobial activity of *Satureja khuzistanica* Essential Oil

	Disc diffusion (mm)	Broth dilution (µg/ml)	
	0.5 µl	MIC	MLC
S. aureus	18.4±0.12	85	170
E. coli	13.8±0.33	170	170
P. aeruginosa	11.5±0.1	340	340
C. albicans	23.4±0.19	43	43
A. niger	27.3±0.61	170	170

MIC=Minimal Inhibitory Concentration; MLC=Minimal Lethal Concentration

Evaluation the preservative efficacy of *S. khuzistanica* essential oil in vegetable oils

The preservative efficacy of *S. khuzistanica* essential oil in vegetable oils was evaluated by evaluating the antimicrobial effectiveness testing. Inoculated vegetable oils and negative controls were contaminated with predetermined number of microorganisms that mentioned above (10^5–10^6 CFU/ml). At time intervals 0, 7, 14, 21, 28 days, the CFU/ml of microorganisms were determined by inserting the aliquots of inoculated vegetable oils into neutralizing broth media at room temperature, then they serially were diluted and were cultured on specified media cultures. The log CFU/ml of each microorganism for each sample were calculated and the results were reported **(Sutton and Porter, 2002)**.

Evaluation the antioxidant activity of *S. khuzistanica* essential oil in vegetable oils

Radical scavenging potency of vegetable oils containing different concentrations of *S. khuzistanica* essential oil (0, 0.25, 0.5, 0.75 and 1% w/w) were determined by free radicals of 1,1-diphenyl-2-picrylhydrazyl (DPPH). Briefly, 40 µl of serial diluted different samples of vegetable oils in methanol was mixed with DPPH. After 70 min incubation period at room temperature, the absorbance of solutions was read against a blank at 517 nm. Inhibition percent of free radicals was calculated and reported. Vegetable oils were used as controls. All experiments were performed in triplicates **(Mahboubi *et al*., 2013)**.

Table 4 The preservative potency of *Satureja khuzistanica* Essential Oil against pathogens in vegetable oils

Sample	*S. aureus*					*E. coli*					*P. aeruginosa*					*C. albicans*					*A. niger*				
Day	0	7	14	21	28	0	7	14	21	28	0	7	14	21	28	0	7	14	21	28	0	7	14	21	28
S	6.3	0	0	0	0	5.9	6.6	6.7	6.8	6.8	6.2	6.2	6.8	6.8	6.4	4.7	4.7	4.8	4.8	4.8	3.4	2.4	2.3	2.3	2.3
SS (0.25%)	6.3	0	0	0	0	5.9	6.5	6.7	6.8	6.8	6.2	5.9	5.9	5.9	5.3	4.7	4.5	4.1	3.9	2.4	3.4	2.4	2	2	1.2
SS (0.5%)	6.3	0	0	0	0	5.7	6.5	6.7	6.7	6.8	6.2	5.6	5.6	5.4	5.2	4.7	4.4	4	3.8	3.6	3.3	1.8	0	0	0
SS (0.75%)	6.3	0	0	0	0	5.9	0	0	0	0	6.2	2.1	0	0	0	4.7	3.3	1.1	0	0	3.3	1.6	0.8	0	0
SS (1%)	6.3	0	0	0	0	5.9	0	0	0	0	6.3	1.0	0	0	0	4.7	2.1	0.7	0	0	3.4	1.1	0.6	0	0
F	6.9	4.7	0	0	0	6.9	6.2	5.9	5.7	5.6	6.2	7.9	8.8	8.6	8.6	4.7	3.9	3.8	3.7	3.7	3.6	2.6	2.5	2.4	2.4
FS (0.25%)	6.8	4.3	0	0	0	6.9	5.7	5.6	5.6	5.2	6.2	7.7	8.7	8.6	8.4	4.7	3.8	3.7	3.6	3.5	3.6	2.3	2.4	2.4	2.2
FS (0.5%)	6.7	4.1	0	0	0	6.7	5.4	5.2	5.1	4.8	6.1	7.7	8.2	7.9	7.6	4.7	3.6	3.5	3.4	3.2	3.6	2.3	2.3	2.3	2.0
FS (0.75%)	6.3	3.8	0	0	0	5.9	0	0	0	0	6.2	4.1	2.8	2.6	2.4	4.7	4.7	3.8	2.8	1.4	3.4	2.4	2.3	2.3	2.1
FS (1%)	6.3	2.8	0	0	0	5.9	0	0	0	0	6.2	3.9	1.8	1.8	1.8	4.7	4.5	3.2	2.1	1.6	3.4	2.4	2	2	1

SS- Sesame oil+*S.khuzestanica* oil, FS- Flaxseed oil+ *S.khuzestanica* oil

RESULTS AND DISCUSSION

Chemical composition of *S. khuzistanica* essential oil

Thirty five different components were identified in *S. khuzistanica* oil, representing 93.2% of total essential oil composition. Carvacrol (84%), β-bisabolene (2.7%), thymol (1%) and p-cymene (0.5%) were the main components of *S. khuzistanica* essential oil **(Tab 1)**.

The effects of *S. khuzistanica* essential oil on chemical profile of vegetable oils

Oleic acid (44.1%), linoleic acid (38.7%), palmitic acid (9.5%) and stearic acid (5.6%) were the major fatty acids of sesame oil while α- linoleic acid (54.9%), oleic acid (19.7%), linoleic acid (13.5%), palmitic acid (5.5%) and stearic acid (5.1%) were the main fatty acids of flaxseed oil **(Tab 2)**.
Inoculation the *S. khuzistanica* essential oil in vegetable oils had no effect on chemical profiles of vegetable oils as the **Table 2** has been shown. In inoculated vegetable oils with essential oil, the amounts of carvacrol were related to the amounts of carvacrol in inoculated essential oil into vegetable oils.

The effects of *S. khuzistanica* essential oil on antioxidant activity of vegetable oils

The antioxidant evaluation of vegetable oils by DPPH assay showed that sesame oil had IC_{50} equal to 26 µg/ml and this IC_{50} was higher than the IC_{50} for flaxseed oil (22 µg/ml). Inoculation of *S. khuzistanica* essential oil in sesame and flaxseed oils increased the antioxidant potencies of inoculated vegetable oils by reduction in IC_{50} of vegetable oils, dose dependently **(Figure 1, Tab 2).** Inoculation the *S. khuzistanica* essential oil (1% v/v) into vegetable oils decreased the IC_{50} of sesame and flaxseed oils from 26, 22 µg/ml to 12 and 7 µg/ml, respectively.

Antimicrobial activity of *S. khuzistanica* oil against microorganisms

The antimicrobial evaluation of *S. khuzistanica* essential oil against tested microorganisms showed that, in disc diffusion method, the most sensitive microorganism was *A. niger* (27.3 mm), followed by *C. albicans* (23.4 mm), and *S. aureus* (18.4 mm), respectively. *E. coli* and *P. aeruginosa* showed the lower inhibition zone diameters than the others (lower than 15 mm).
Antimicrobial evaluations of *S. khuzistanica* essential oils by micro broth dilution assays exhibited the microorganisms had different behavior in broth media in comparison with solid media. *C. albicans* with MIC and MBC values of 43 µg/ml exhibited more sensitivity to *S. khuzistanica* oil, followed by *S. aureus* (MIC, MLC= 85, 170 µg/ml). *A. niger* and *E. coli* had the same sensitivity to *S. khuzistanica* oil. *P. aruginosa* was the less sensitive microorganisms to *S. khuzistanica* oil (MIC, MLC= 340 µg/ml) **(Tab 3)**.

The preservative potency of *S. khuzistanica* essential oil in vegetable oils

Sesame and flaxseed oils alone inhibited the growth of *S. aureus*, therefore, inoculation of *S. khuzistanica* essential oil in vegetable oils for inhibition the growth of *S. aureus* was meaningless. The log CFU/ml of other bacteria such as *E. coli* and *P. aeruginosa* in vegetable oils did not inhibited by vegetable oils after 28 days. The log CFU/ml of *P. aeruginosa* particularly increased. Inoculating the essential oil (0.25% and 0.5% v/v) into vegetable oils did not exhibit any preservative effects against bacteria and molds. Sesame oil with *S. khuzistanica* essential oil at concentration of 0.75% and 1% (v/v) completely inhibited the growth of tested microorganisms. Flaxseed oil also inhibited the growth of *E. coli* after 7 days in presence of 0.75% (v/v) of *S. khuzistanica* essential oil but the log CFU/ml of *P. aeruginosa*, *C. albicans* and *A. niger* decreased to 2.4, 1.4 and 2.1 after 28 days in inoculated vegetable oil with *S. khuzistanica* essential oil (0.75% v/v). *P. aeruginosa* was the most resistant microorganisms to inoculated vegetable oils **(Tab 4)**.
As the results are shown, carvacrol was the main components of *S. khuzistanica* essential oil according to the results of other investigators **(Saidi, 2014; Siavash Saei-Dehkordi *et al.*, 2012)**. Furthermore, the antimicrobial activity of *S. khuzistanica* essential oil **(Akbari-Shahabi *et al.*, 2014; Siavash Saei-Dehkordi *et al.*, 2012)**, carvacrol (**Cacciatore *et al.*, 2015**) and the mechanism of action for carvacrol **(Ait-Ouazzou *et al.*, 2013; Chueca *et al.*, 2014)** have been confirmed. Also, the antioxidant activity of *S. khuzistanica* essential oil has been reported (**Ahmadvand, 2014**). Although, there are many investigations on biological activities of *S. khuzistanica* essential oil but to now, there is no study that evaluates the preservative and antioxidant potency of *S. khuzistanica* essential oil in valuable vegetable oils with therapeutic potencies. Nowadays, the use of natural products among the consumers has increased and natural products have good features in different part of the World. Therefore, finding the natural preservatives with other pharmacological potency such as anti-inflammatory and analgesic effects (**Esmaeili-Mahani *et al.*, 2015**) can enhance the therapeutic potencies of vegetable oils, meanwhile it can protect vegetable oils against deterioration. *S. khuzistanica* essential oil by preservative and antioxidant potency protects sesame and flaxseed oils from deterioration and also enhances the therapeutic potency of these oils.

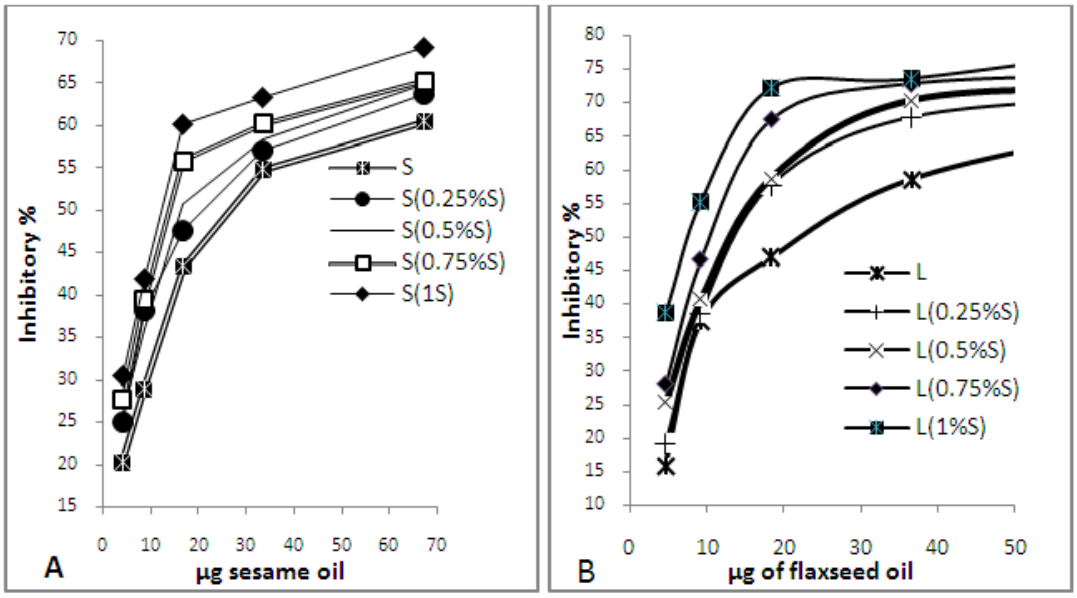

Figure 1 The antioxidant activity of Sesame (A), flaxseed (B) oils with different concentration of *S. khuzistanica* oil by DPPH method

According to our results, sesame oil has shown the better antimicrobial potency than that of flaxseed oil, while its antioxidant activity was lower than the flaxseed oil. The difference in antimicrobial and antioxidant activities of vegetable oils is related to their fatty acid profiles. Oleic acid, linoleic acid (unsaturated fatty acids) as the main components of sesame oil have shown the antimicrobial activity against Gram positive bacteria with MIC 0.01-0.1 mg/ml and also they have shown the synergistic effects with each other (**Dilika *et al.*, 2000**). In fact, the inhibition of *S. aureus* growth by vegetable oils particularly by sesame oil is related to oleic acid, linoleic acid and their synergistic effects between oleic acid and linoleic acid.
Furthermore, it has been reported, among different identified fatty acids in vegetable oils, palmitic acid and stearic acid has exhibited a weak antibacterial activity against *S. aureus*, while oleic acid and linoleic acid has shown high antibacterial activity against *S. aureus*. The antibacterial activity of oleic acid has been reported higher than that of linoleic acid (**Zheng *et al.*, 2005**). In fact, the presence of higher antibacterial agents in sesame oil makes it as stronger antimicrobial agents. In total, identified unsaturated fatty acids in sesame oil were

83% of total fatty acids, while the corresponding amounts in flaxseed oil were 88%. Identified saturated fatty acids were 15.1 and 10.6% for sesame and flaxseed oils, respectively. The interaction between saturated and unsaturated fatty acids makes flaxseed oils as a valuable antioxidant, although, the antioxidant potency of sesame oil was good. Other valuable finding of our study was traceability the essential oil in vegetable oils. In other word, carvacrol did not have any effects on chemical profiles of vegetable oils and the amount of carvacrol in vegetable oils was related to the inoculated essential oil into vegetable oil.

CONCLUSION

In conclusion, the use of *Satureja khuzistanica* essential oil in sesame and flaxseed oils as preservative and antioxidant agents can protect the vegetable oils from deterioration and also donates it other biological activity such as anti-inflammatory effect and analgesic potency, without any changes in fatty acid profile of vegetable oils, whereas the use of this natural agent help to remove the adverse effects of chemical antioxidant and antimicrobial agents from the life of humans. The limitation of our study was no assessing the organoleptic effects of *S. khuzistanica* in vegetable oils for oral applications.

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