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PYRENE BIODEGRADATION POTENTIALS OF AN ACTINOMYCETE, *MICROBACTERIUM ESTERAROMATICUM* ISOLATED FROM TROPICAL HYDROCARBON-CONTAMINATED SOIL

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ABSTRACT

A novel pyrene-degrading actinomycete, phylogenetically identified as *Microbacterium esteraromaticum* strain SL9 was isolated from a polluted hydrocarbon-contaminated soil in Lagos, Nigeria. Growth of the isolate on pyrene was assayed using total viable counts, pyrene degradation was monitored using gas chromatography (GC-FID) while UV-Vis spectrophotometry was used to detect metabolites of pyrene degradation. The isolate tolerated salt concentration of up to 6%, grew luxuriantly on crude oil and exhibited weak utilization of fluorene, acenaphthene and engine oil. It resisted cefotaxime, ciprofloxacin and amoxicilin, but was susceptible to meropenem, linezolid and vancomycin. It also resisted elevated concentrations of heavy metals such as 1-5 mM lead and nickel. On pyrene, the isolate exhibited growth rate and doubling time of 0.023 h^{-1} and 1.25 h, respectively. It degraded 55.16 (27.58 mg L^{-1}) and 89.28% (44.64 mg L^{-1}) of pyrene (50 mg L^{-1}) within 12 and 21 days respectively, while the rate of pyrene utilization was 0.09 mg $L^{-1}h^{-1}$. Catechol dioxygenase assay using UV-Vis spectrophotometry revealed the detection of *meta* cleavage compound, 2-hydroxymuconic semialdehyde in the crude cell lysate. The results of this study showed the catabolic versatility of *Microbacterium* species on hydrocarbon substrates and their potential as seeds for bioremediation of environments co-contaminated with polycyclic aromatic hydrocarbons and heavy metals.

Keywords: Biodegradation; pyrene; Hydrocarbon-contaminated soils; *Microbacterium esteraromaticum*

INTRODUCTION

Pyrene is a peri-condensed four-ring polycyclic aromatic hydrocarbon (PAH). It belongs to the class of non-alternant high molecular weight (HMW) PAH. It is a regulated pollutant at sites contaminated with petroleum. Pyrene equally results from incomplete combustion of organic materials and other processes of pyrolysis and pyrosynthesis (**Kanaly and Harayama, 2010**). Like all HMW PAHs, pyrene exhibits high molecular stability, low solubility in aqueous phase and is highly hydrophobic, thus accounting for its persistence in the environment. Although not known to be carcinogenic, its mutagenicity has been demonstrated in laboratory animals and it is equally known to be highly toxic with evidence of respiratory function impairment (**USDHS, 1990**). It is a signature compound for the study of carcinogenic high molecular weight polycyclic aromatic hydrocarbons degradation such as benzo(a)pyrene with which it shares structure (**Obayori *et al*., 2013**)

The first pyrene degrading bacterium to be isolated was *Mycobacterium* sp. Strain PYR1 (**Heitkamp *et al*., 1988a**) later renamed correctly as *Mycobacterium vanbaalenii* strain PYR1 by **Khan *et al*. (2002)**. Subsequently, more pyrene degraders, mainly norcadioform actinomycetes with remarkable metabolic versatility were reported (**Walter *et al*., 1991; Boldrin *et al*., 1993; Schneider *et al*., 1996; Dean-Ross and Cerniglia, 1996; Churchill *et al*., 1999; Bastiaens *et al*., 2000**). But today, the literature is replete with pyrene degraders spanning low G+C Gram-positive and Gram-negative genera such as *Bacillus*, *Corynebacterium, Micrococcus, Pseudomonas, Sphingomonas, Stenotrophomonas, Alcaligenes, Achromobacter, Leclercia, Cycloclasticus, Burkholderia* and *Proteus* (**Sarma *et al*., 2004; Gaskin and Bentham, 2005; Lin and Cai, 2008; Wang *et al*., 2008; Ceyhan, 2012**). Notwithstanding the rich literature on pyrene degradation globally, reports of HMW PAH degraders from sub-Sahara Africa are virtually absent. However, **Obayori et al. (2008)** reported the isolation of three pyrene degrading *Pseudomonas* strains from hydrocarbon-contaminated soils in Lagos, Nigeria.

Actinomycetes are a group of diverse bacteria that are Gram positive with high guanine plus cytosine in the DNA (> 55 mol %). They are good candidates for bioremediation of polluted soils due to their capablility to produce extracellular enzymes that degrade a wide range of complex organic compounds, production of spores that are impervious to desiccation and filamentous growth that favours colonization of soil particles (**Ensign, 1992; Salam *et al*., 2014**). Particularly, actinomycetes are good candidates for degradation of hydrophobic compounds such as PAHs due to their surfactant-producing activity. The biosurfactant may be extracellular such as glycolipids (trehalose lipid and lipopeptide produced by *Rhodococcus* and *Arthrobacter* species) or cellular biosurfactants such as mycolic acid, which allow adherence of the microbial cells to hydrophobic phases in two-phase systems (**Singer and Finnerty, 1990; Morikawa *et al*., 1993; Neu, 1996**).

The initial reactions in the classic aerobic degradation of pyreneby bacteria are catalysed by a multi-component enzyme system and involve the incorporation of other atoms of oxygen molecules at the 4, 5-carbon positions to produce a dihydrodiol. The dihydrodiol is re-aromatised and the dihydroxylated product is cleaved to products, which are processed through the phenanthrene pathway into the tricarboxylic acid (TCA) cycle (**Heitkamp *et al*., 1988b**). However, a plethora of other routes exist, some leading to complete degradation, while others merely lead to dead-end products (**Kim *et al*., 2007**). The significance of pyrene in the bioremediation of petroleum-polluted sites and the effect of products of incomplete degradation of pyrene on the fate of other PAHs in the environment has further compelled research on pyrene degraders (**Kazunga and Aitken, 2000**).

Heavy metals are introduced into soil compartments through various anthropogenic activities such as petroleum exploration, waste disposals, and corrosion of metals in use among others. Toxicity of heavy metals to autochthonous microbial community in soil has been reported to also inhibit biodegradation of pollutants in co-contaminated sites (**Sandrin *et al*., 2000**). To tolerate heavy metals stress, microorganisms have evolved various resistance mechanisms such as efflux pumps, enzymatic detoxification, permeability barriers, intra- and extracellular sequestration, and reduction (**Nies, 1999**).

Notwithstanding recent shift in focus from isolation to demonstration of degraders in the environment in recent years, considerable attention continues to be directed to search for novel culturable organisms. Such isolates are continuously relevant in discerning relationship between phylogeny and specific metabolic function and also for the purpose of bio-augmentation (**Hilyard *et al*., 2008**). Previously, we had reported the degradation of anthracene by a *Microbacterium* species (**Salam *et al*., 2014**). Here we report the isolation and characterization of a *Microbacterium esteraromaticum* strain able to use pyrene as sole source of carbon and energy. To the best of our knowledge, this is the first report of isolation and characterization of pyrene-degrading *Microbacterium esteraromaticum* strain.

MATERIALS AND METHODS

Sampling site

Soil samples for this study were collected from an automobile workshop at Okokomaiko, Lagos, Nigeria. The coordinates of the sampling site were latitude 6° 28' 23″ N and longitude 3° 11' 14″ E, respectively. Soil samples were collected at a depth of 10–12 cm using sterile trowel after clearing debris from the soil surface. Samples for physicochemical analysis were collected in clean black polythene bags, while samples for microbiological analysis were collected in sterile screw-capped bottles. Immediate analysis of the samples were carried out within 5 h of collection or stored at 4 °C. Physicochemical properties of the soil sample, which has been described elsewhere showed a weakly acidic pH of 6.10, an unusually high hydrocarbon content (157,068 mg/kg) and a plethora of heavy metals such as lead (0.11 mg/kg), zinc (3.31 mg/kg) and nickel (4.34 mg/kg) (**Salam *et al*., 2014**).

Enrichment and isolation of pyrene-degrading bacteria

Bacteria with capacity for pyrene degradation were isolated on pyrene mineral salts medium (MSM) by continual enrichment method. Mineral salts medium described by **Mills et al. (1978)** and modified by **Okpokwasili and Amanchukwu (1988)** was used. The medium was supplemented with yeast extract (0.005 g/l) as source of growth factors. After adjusting the pH to 7.0, the medium was fortified with 50 µg/ml and 20 µg/ml of nystatin and nalidixic acid respectively for selective isolation of actinomycetes. Air-dried contaminated soil (5 g) was added to 45 ml of MSM containing 50 mg/l of pyrene. Enrichment was carried out by incubation with shaking (180 rpm) at room temperature (29 ± 2 °C) in the dark for 4–5 weeks until there was turbidity. After five consecutive transfers, pyrene degraders were isolated by plating out dilutions from the final flasks on Luria–Bertani (LB) agar. The colonies that appeared were further purified by subculturing once onto LB agar. Ability to degrade pyrene was confirmed by inoculating phosphate buffer-washed LB broth grown culture in fresh MSM flask supplemented with 50 mg/l pyrene as sole carbon source. One isolate designated strain SL9 out of the six screened was selected for further study based on its extensive degradative ability.

Maintenance and identification of isolates

The pure pyrene-degrading isolate was maintained in glycerol/LB broth medium (1:1, v/v). Pure colonies subcultured on LB agar supplemented with low percentage of pyrene (0.005%) were harvested with sterile inoculating loop, pooled and transferred to the medium. The mixture was shaken to homogenize and kept at -20°C.

Identification and characterization of isolate

Pure culture of bacterial isolate was identified based on their colonial morphology, cellular morphology and biochemical characteristics according to the identification scheme of Bergey's Manual of Determinative Bacteriology (**Holt *et al*., 1994**). Antibiotic susceptibility of the isolate was carried out using multidiscs. The antibiotics tested include streptomycin (30 µg), erythromycin (15 µg), vancomycin (30µg), doxycycline (30µg), meropenem (10µg), linezolid (30 µg), cefotaxime (30 µg), ciprofloxacin (5 µg), amoxicillin (30 µg) and pefloxacin (30 µg). Salt tolerance was tested in LB broth supplemented with NaCl ranging from 1 - 10% (w/v). Incubation was carried out at room temperature (29 ± 2°C) for one week with shaking and daily observation for growth. PCR amplification of the 16S rRNA gene from genomic DNA of strain SL9 was performed using the universal primers 27f (5'-AGAGTTTGATC{A/C}TGGCTCAG-3') and 1378r (5'-CGGTGTGTACAAGGCCCGGGAACG-3') (**Heuer *et al*., 1997**). The reaction mix contained 20 pmol each of universal primers, 10 µl of Ex *Taq* buffer (Mg^{2+} plus), 2.5 mM of each dNTPs, 2.5 U (0.5 µl) of Ex *Taq* polymerase (Takara) and 1.0 µl of purified genomic DNA in a total volume of 100 microlitres. Amplification conditions consisted of an initial denaturation step at 95°C for 3 min, 30 amplification cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 2 min with the final extension at 72°C for 7 min before cooling to 4°C.

The amplicon was analyzed on 1% agarose gel in 1xTAE, run at 100V for 30-35 min. The PCR product (~1500 bp DNA fragment) was cloned into the plasmid vector pT7Blue® (Novagen, USA) after purification by centrifugation using Wizard® SV Gel and PCR Clean-up System (Promega, Madison, Wis.). The purified PCR product was transformed into *Escherichia coli* strain DH5α (Toyobo). After the transformation of *E. coli* DH5α-competent cells, clones were picked. Selected clones were then sequenced using the ABI Prism 3730xl DNA Sequencer (Applied Biosystems, UK) according to the manufacturer's instructions. The 16S rRNA nucleotide sequence obtained from both strands was aligned (CLUSTAL W) and the homology search for 16S rRNA was performed in the DDBJ/EMBL/GenBank database using the Basic Local Alignment Search Tool (BLAST) program (**Altschul *et al*., 1990**). Strain SL9 sequence had been deposited in the DDBJ/EMBL/GenBank database under the accession number AB646581.2. Phylogenetic tree showing evolutionary relationship of strain SL9 with reference sequences from the database was constructed using neighbour joining algorithm within the program MEGA 5.1 (The Biodesign Institute) and bootstrapped with 100 repetitions.

Substrate Specificity of strain SL9

Substrates utilization pattern of strain SL9 on different hydrocarbons was evaluated in MSM containing the respective hydrocarbons as sole carbon and energy source at a concentration of 100 ppm. Stock solution of each hydrocarbon substrate was prepared by dissolving 1 g of it in 10 ml dimethyl sulfoxide (DMSO) and filter-sterilizing the solution using hydrophobic filter (Advantec, JP020AN). Sterile MSM (5 ml) was prepared in test tubes and 5 µl of stock solution of the respective hydrocarbons was added. Strain SL9 was added at 1% (v/v). Incubation was carried out at room temperature in the dark for 14 days. Test tubes prepared as above but without carbon source served as control. Degradation was monitored by visual observation for turbidity. The hydrocarbons tested include naphthalene, fluorene, acenaphthene, pyrene, dibenzothiophene and dibenzofuran. Liquid hydrocarbons like crude oil and engine oil were autoclaved and added separately to the sterile MSM at 0.1% (v/v).

Metal tolerance assay

Strain SL9 was grown in LB broth for 18 h at room temperature. Cells were harvested by centrifugation (7,000×g; 10 min), washed twice with sterile phosphate buffer, and resuspended in the same buffer solution. The cell concentration of bacterial suspensions was determined by measuring the optical density of the samples at 600 nm and relating the value to a calibration curve (10^{10}cfu L^{-1}=1 ODunit). Stock solutions (1 M) of metal salts namely, $HgCl_2$, $NiSO_4$, and $Pb(NO_3)_2$ were prepared in distilled water, filter sterilized using 0.22-µm membrane filters, and stored in sterile bottles in the dark at 4 °C. Dilutions to 1, 5, 10, and 15mM of Hg^{2+}, Ni^{2+}, and Pb^{2+}were made from the stock solutions into LB broth. The media were dispensed in 5-ml aliquots and inoculated with 50 µl (1%, v/v) inoculum. Each of the experiment was conducted in triplicates. LB broth not supplemented with heavy metals and inocula serves as controls. Growth of the inocula was measured by absorbance at 600 nm and occasional viable count assay. Resistance was assayed by determining the maximum tolerance concentrations (MTCs) for the isolates after 10 days of incubation. MTC is defined as the highest concentration of metal, which do not affect the viable counts of organisms.

Evaluation of pyrene biodegradation

Pyrene degradation potentials of the pure isolate was assayed by inoculating 250-ml replicate flasks containing 50 ml of MSM supplemented with 50 mg/l of pyrene as sole carbon and energy source respectively. Flasks were inoculated with 0.5 ml of MSM-washed 18 h-24 h LB agar-grown cells and subsequently incubated at 180 rpm in the dark for 21 days at room temperature. Flask containing heat-killed cells sterilized at 121°C for 15 min and supplemented with pyrene as described above were used as controls. Samples were withdrawn from each flask at 3 days interval and aliquots of appropriate dilutions were plated (in triplicates) onto nutrient agar for total viable counts (TVC).

Extraction of residual pyrene

Residual pyrene was extracted by liquid-liquid extraction. Briefly, broth culture (50 ml) was extracted twice with an equal volume of hexane. After removing the aqueous phase with separating funnel, the organic fraction was concentrated to 1 ml and the residual pyrene concentration was determined by gas chromatography. Control flasks were also extracted similarly.

Analytical method

Hexane extracts (1.0 µl) of residual pyrene were analyzed with Hewlett Packard 5890 Series II gas chromatograph equipped with flame ionization detector (FID) and 30 m long HP-5 column (internal diameter, 0.25 mm; film thickness, 0.25 µm). The carrier gas was nitrogen. The injector and detector temperatures were

maintained at 300°C and 320°C, respectively. The column temperature was programmed from 60°C to 500°C for 27 min. The gas chromatograph column was programmed at an initial temperature of 60°C; this was held for 2 min, and then ramped at 12°C/min to 205°C and held for 16 min. nitrogen column pressure was 37 psi, the hydrogen pressure was 9 psi and compressed air pressure was 13 psi. The software was Chem Station. Rev. A. 05. 01.

Catechol dioxygenase assay

Two milliliters of strain SL9 was harvested by centrifugation at the late logarithmic phase from MSM medium containing pyrene and was suspended in1-ml MSM. Cells were lysed by the addition of 20 µl toluene and after vigorous mixing, unbroken cells and cell debris were removed by centrifugation at 16,000×g for 30 s. The clear supernatants were immediately used for the assay or placed on ice for not more than 10 min. Activity assays were performed using GENESYS 10S UV-Vis spectrophotometer (Thermo Scientific, USA). The reaction was initiated by the addition of 100 µl catechol solution (100 µM) to a reaction mixture in a 1-cm light path quartz cuvette containing 800 µl phosphate buffer and 100 µl of crude lysate. The blank cuvette contained the same amount of enzyme in the same buffer with the exception of catechol. Activities of catechol 1,2-dioxygenase and 2,3-dioxygenase were monitored at 260 and 375 nm, respectively (**Wang *et al*., 2008**).

Statistical analysis

Mean generation times (T_d) and specific growth rates (µ) of the isolate on pyrene was calculated using non-linear regression of growth curves for the period when growth rates were maximal using Prism version 5.0 (Graphpad software, San Diego, CA).

RESULTS

Identification and characterization of pyrene-degrading isolate

Continuous enrichment resulted in the isolation of several pyrene degraders. However, only one isolate designated strain SL9 out of the six isolates, displayed strong degradative ability. Phenotypic characterization of the isolate showed that it was obligately aerobic, Gram-positive, non-spore forming, irregular rods occurring singly or in clusters. On LB agar, strain SL9 was circular, smooth, translucent, yellow-pigmented, opaque, low-convex, moist colonies with entire margins. Strain SL9 colonies were catalase positive but showed negative reaction for oxidase, methyl red, Voges-Proskauer, indole, gelatinase and H_2S production. It was positive for starch hydrolysis and is unable to utilize all the sugars tested with exception of mannitol, maltitol and salicin. The isolate tolerated salt concentration of up to 6% while growth cease above this concentration. It resisted cefotaxime, ciprofloxacin and amoxicillin, but was susceptible to meropenem, linezolid and vancomycin.

However, comparisons of the 16S rRNA partial fragments of strain SL9 (1374 bp) with the nucleotide sequences in the DDBJ/EMBL/GenBank database indicated significant alignments of the strain with *Microbacterium esteraromaticum*. Nucleotide sequence analysis of the partial 16S rRNA gene showed that Strain SL9 exhibited 99% identity with other *Microbacterium esteraromaticum* strains such as *M. esteraromaticum* strain SL6 (AB646579.2), and *M. esteraromaticum* strain S29 (AB099658.1), respectively. The relationship between strain SL9 (AB646581.2) and the nearest phylogenetic relatives is shown in Figure 1.

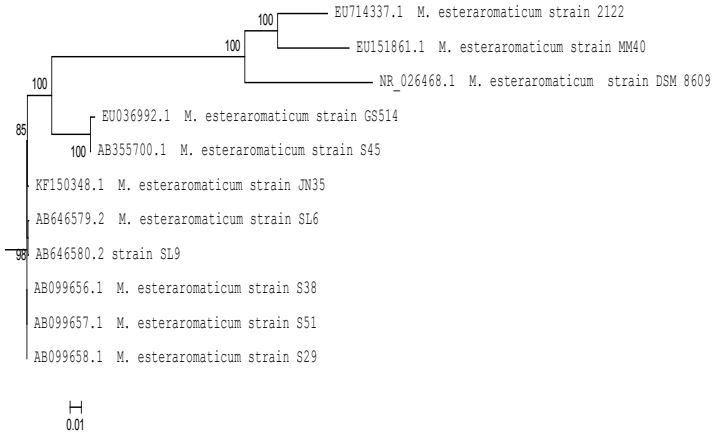

Figure 1 Phylogenetic tree resulting from neighbour joining (NJ) analysis of 16S rRNA showing the phylogenetic positions of pyrene-degrading *Microbacterium esteraromaticum* strain SL9 (AB646580.2) and related strains of *M. esteraromaticum* retrieved from NCBI GenBank. Accession number of each microorganism used in the analysis is shown before the species name. The scale bar indicates the numbers of nucleotide substitutions per position. Bootstrap values (expressed as percentage of 100 replicons) are shown at the branch.

Substrate specificity of pyrene-degrading isolate

Substrate spectrum analysis of strain SL9 on various aromatic and heteroaromatic hydrocarbon substrates was conducted to ascertain the substrate utilization pattern of the isolate. The analysis revealed different utilization patterns. Strain SL9 failed to grow on naphthalene and dibenzofuran. It grew luxuriantly on pyrene and crude oil but exhibited weak growth on fluorene, acenaphthene and engine oil, respectively (Table 1).

Table 1 Substrate specificity test

Substrate	Isolate (SL
Naphthalene	-
Fluorene	++
Acenaphthene	++
Pyrene	+++
Dibenzofuran	-
Dibenzothiophene	+
Crude oil	+++
Engine oil	++

+++ Luxuriant growth (>10^6 cfu/ml after 5 days of incubation); ++ Weak growth (>10^6 cfu/ml after 1 week of incubation); + Poor growth (>10^6 cfu/ml after 2 weeks of incubation); - No growth (<10^6 cfu/ml after 2 weeks of incubation)

Metal tolerance of pyrene-degrading isolate

Metal tolerance assay of strain SL9 on various heavy metals was conducted to determine the tolerance limit of the isolate to various concentrations of heavy metals. The assay revealed different resistance patterns of the isolate to each of the heavy metal. Cessations of growth were observed in the presence of 5, 10, and 15 mM mercury as well as 10 and 15 mM lead and nickel. However, the isolate showed resistance to 1 mM mercury and also resisted 1–5 mM of nickel and lead, respectively.

Kinetics of pyrene degradation

The utilization of pyrene as a sole source of carbon and energy by strain SL9 was confirmed by an increase in cell population with a concomitant loss of pyrene. The growth kinetics of strain SL9 on pyrene is depicted in Figure 2 and Table 2. The strain exhibited a short lag phase followed by consistent increase in cell density with corresponding decrease in pyrene concentration. Strain SL9 grew exponentially from an initial cell density of 1.85 x 10^6 cfu/ml to 2.81 x 10^8 cfu/ml in 9 days. The growth decline to 2.76 x 10^8 cfu/ml at day 12 but peaked slightly at day 15 to 2.79 x 10^8 cfu/ml. It thereafter maintained a decreasing trend. During the exponential growth of the isolate on pyrene, it exhibited a growth rate and doubling time of 0.023 h^{-1} and 1.25 h, respectively.

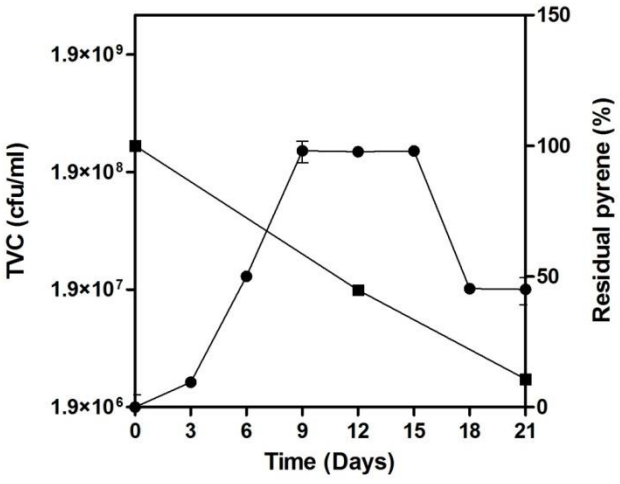

Figure 2 Growth dynamics of strain SL9 in minimal medium amended with 50 mg/l pyrene showing total viable count, TVC (●) and residual pyrene (■). Data points represent the mean of three replicate flasks. In the case of population counts, error bars represent standard deviation. Residual pyrene was determined with reference to pyrene recovered from heat-killed controls.

Pyrene transformation by *M. esteraromaticum* strain SL9 was studied at 72-h intervals in MSM containing 50 mg/L pyrene. Gas chromatographic analysis of residual pyrene showed that after 12 days of incubation, pyrene content decreased to 44.84% (22.42 mg/L) corresponding to removal of 55.16% (27.58 mg/L) pyrene. At the end of 21 days incubation, the residual pyrene content decreased further to 10.72% (5.36 mg/L) corresponding to uptake of 89.28% (44.64 mg/L) pyrene. However, in the heat-killed control flasks, no apparent decrease of the substrate was observed, thus confirming that pyrene depletion from the MSM was due to biodegradation by strain SL9 rather than to non-specific abiotic losses such as substrate volatility or absorption to the glass tubes.

Table 2 Growth kinetics of pyrene-degrading isolate

Isolate	Growth rate, K (h^{-1})	Mean generation time, ΔT_d (h)	% degradation[1] (day 12)	% degradation[1] (day 21)	Degradation rate (%/h)	Rate of degradation (mg $L^{-1}h^{-1}$)
Microbacterium esteraromaticum strain SL9	0.023	30.0	55.16	89.3	0.18	0.09

[1]Percent degradation values represent the net decrease (FID area counts) calculated with reference to the amount recovered from heat-killed control flasks

Detection of ring fission enzyme

Strain SL9 degraded catechol via the *meta* pathway as reflected in increase activity (increase in absorbance spectra values) at 375 nm when monitored using UV-Vis spectrophotometer. This indicate the formation of 2-hydroxymuconic semialdehyde via catechol 2,3-dioxygenase activity (Figure 3). At absorbance value of 260 nm, there is consistent decrease in absorbance spectra values, indicating that the isolate lacks the ability to degrade catechol using the *ortho* pathway.

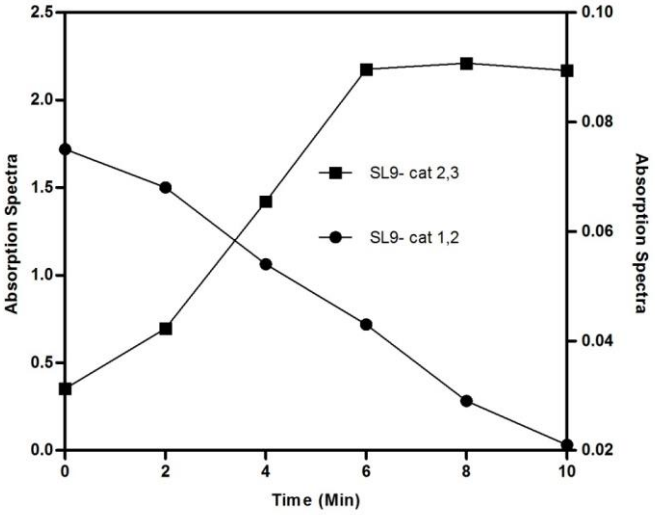

Figure 3 Enzymatic transformation of catechol to 2-hydroxymuconic semialdehyde by lysate of pyrene-grown cells. The reaction was started in a sample cuvette containing 100 ml of cell lysate in 800 ml of phosphate buffer, pH 7.5, by the addition of 100 mM catechol. Optical absorption spectra were recorded at periodic intervals of 0, 2, 4, 6, 8, and 10 min. Increase in absorption spectra at 375 nm indicate conversion of catechol to 2-hydroxymuconic semialdehyde by strain SL9. Consistent decreases in absorption spectra were observed at 260 nm.

DISCUSSION

PAHs are ubiquitous environmental pollutants renowned for their hydrophobicity, low bioavailability and bioaccumulation potential (**Nkansah *et al*., 2011**). A good knowledge of biodegradative potentials of novel strains isolated from contaminated sites is pivotal in designing an enduring bioremediation strategy. In this study, pyrene, a peri-condensed PAH was degraded by a Gram-positive, high G + C actinomycete, *Microbacterium esteraromaticum* sp. strain SL9, isolated from a heavily polluted hydrocarbon contaminated soil. Previously, **Obayori et al. (2008)** isolated three pyrene-degrading *Pseudomonas* spp. strains LP1, LP5 and LP6 from hydrocarbon-contaminated soil in Lagos, Nigeria.

The isolate tolerated salt concentration of up to 6%. This physiological property qualifies strain SL9 as a possible candidate for bioaugmentation purposes. It has been demonstrated that salinity of the inoculating medium could be crucial in the survival and proliferation of allochthonous strains during bioremediation process (**Kastner *et al*., 1998**). Microorganisms produce antibiotics as a survival strategy and for competitive edge in an oligotropic, highly compacted and diverse contaminated soil environment. Therefore, adequate understanding of antibiotic sensitivity and resistant patterns of isolates with potentials as seed for bioremediation is crucial. Resistance of strain SL9 to amoxicilin, cefotaxime and ciprofloxacin may be attributed to acquisition of resistant genes to these antibiotics through gene transfer, as soil environments are replete with these antibiotics, which could allow evolution of resistance by indigenous strains.

The substrate utilization pattern of strain SL9 on various aromatic and heteroaromatic hydrocarbon substrates revealed different utilization patterns. This may be attributed to the varied composition of the substrates and the diverse nature of hydrocarbon products present at the site where the isolate was recovered.The luxuriant growth of the isolates observed on crude oil as compared to sparse growth on engine oil may be attributed to two factors. First, crude oil, a complex mixture of different chemical composition may favorably support growth of microorganisms better than refined petroleum product such as engine oil due to diverse nutrient options available in crude oil as source of carbon and energy. Second, at the MWO site where the isolate was recovered, different types of oil products may have been indiscriminate disposed. These pollutants inevitably found their way into the soil thereby resulting in adaptation of autochthonous organisms to the pollutants due to selective pressure and acquisition of degradative abilities (**Salam *et al*., 2011**).

In this study, the isolate tolerated 1 mM mercury and 1-5 mM nickel and lead, respectively. This is not surprising considering the detection of iron, lead, zinc and nickel in the soil as revealed in the soil physicochemistry. The presence of these heavy metals at the sampling site indicate gross pollution as heavy oils and spent oils rich in heavy metals are indiscriminately disposed at the sampling site. Isolation of a pyrene degrader from MWO sampling site in spite of the high heavy metals presence may be due to the possibility of the degrader harboring genes for heavy metals resistance and the high hydrocarbon and organic carbon contents of the site. Heavy metals are persistent environmental pollutants that are introduced into the environment through anthropogenic activities and other sources of industrial wastes. In low concentrations, heavy metals are micronutrients, which play significant roles in cell growth and metabolic functions. However, at high concentrations, heavy metals induce oxidative stress, interfere with protein folding, and function (**Nies, 1999**).

Strain SL9 grew on pyrene with a growth rate and doubling time of 0.023 h^{-1} and 1.25 h, respectively. This growth rate is lower than 0.056 h^{-1} reported by **Boldrin et al. (1993)** for *Mycobacterium* BB1. However, this value is higher than 0.014 and 0.013 h^{-1} reported by **Thibault et al. (1996)** for *Pseudomonas* spp. K-12 and B-24, respectively. It is equally higher than 0.018 and 0.017 reported by **Obayori et al. (2008)** for *Pseudomonas* spp. LP1 and LP6, respectively. In addition, pyrene utilization rate of strain SL9 (0.09 mg l^{-1} h^{-1}) is lower than 0.56 mg l^{-1} h^{-1} reported by **Dean-Ross and Cerniglia (1996)** for *Mycobacterium flavescens* and 0.111 mg l^{-1} h^{-1} reported by **Obayori et al. (2008)** for *Pseudomonas* sp. LP1. It is however higher than 0.082 and 0.067 mg l^{-1} h^{-1} reported by the latter for *Pseudomonas* spp. LP5 and LP6, respectively. Earlier observations have indicated that growth rate of isolates on substrates and their rate of utilization is not entirely determined by the intrinsic properties of the isolates but also culture conditions such as crystal size of substrates and variations in physicochemical parameters such as pH (**Grosser *et al*., 1991; Boldrin *et al*., 1993**).

In this study, strain SL9 degraded 55.16 and 89.24% of the initial concentration of pyrene (50 mg l^{-1}) in 12 and 21 days. This degradation rate is lower than 72% (500 mg l^{-1}) in two weeks and 62% in 24 h reported for *M. flavescens* and *Rhodococcus* strain UW1, respectively (**Walter *et al*., 1991; Dean-Ross and Cerniglia, 1996**). It is however higher than 61.5% reported for *Leclercia adecarboxylata* (Sarma ***et al*., 2004**). It is also higher than 65.8% and 33.7% (50 µg ml^{-1}) within 21 days reported for *Bacillus cereus* Py5 and *Bacillus megaterium* Py6 as well as 68, 67 and 47% within 30 days reported for *Pseudomonas* spp. Strains LP1, LP5 and LP6, respectively (**Lin and Cai, 2008; Obayori*et al*., 2008**).

Aerobic degradation of PAHs normally proceeds via the upper pathway characterized by ring destabilization and cleavage through dioxygenation and dehydrogenation, resulting in a catecholic intermediate (**Mason and Cammack, 1992; Diaz, 2004; Vaillancourt *et al*., 2006**). In the lower pathway, ring fission of the catecholic intermediate through the *ortho* (catechol 1,2- dioxygenase) or *meta* cleavage (catechol 2,3 dioxygenase) occurs, which lead to intermediates of the tricarboxylic acid cycle. In this study, catechol is *meta* cleaved by catechol 2,3dioxygenase (C23O) produced by strain SL9 resulting in the formation of 2-hydroxymuconic semialdehyde. Though **Obayori et al. (2008)** reported the detection of catechol 1,2 dioxygenase in three pyrene-degrading *Pseudomonas* strains isolated from contaminated soils in Lagos, Nigeria, several researchers have reported the ubiquity of C23O in most PAH degradation pathway (**Meyer *et al*., 1999; Zhao *et al*., 2011**). This is partly due to their versatility and their

preponderance in many catabolic and biosynthetic pathways (**Vaillancourt *et al*., 2006**).

CONCLUSION

This study described, for the first time, pyrene biodegradation potential of a *Microbacterium esteraromaticum* strain from tropical hydrocarbon-contaminated soil and also showed the degradative ability of the isolate on other aromatics and complex hydrocarbon mixtures. This study also demonstrate for the first time the ability a pyrene-degrading *Microbacterium esteraromaticum* strain to resist some antibiotics and tolerate elevated concentrations of some heavy metals. This showed its potential as seed for bioremediation of hydrocarbon-impacted soils co-contaminated with heavy metals. Further research works will focus on the metabolic pathway employed by strain SL9 for pyrene degradation and the degradative genes involved in the process.

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EVALUATION OF BIOCONVERSION CONDITIONS ON REUTERIN PRODUCTION USING RESPONSE SURFACE METHODOLOGY AND *LISTERIA MONOCYTOGENES* AS TARGET BACTERIA

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ABSTRACT

The objective of this study was to evaluate the effect of bioconversion conditions on reuterin production by *Lactobacillus reuteri* using response surface methodology (RSM). A central composite design consisted of three independent factors at 5 levels: concentration of *Lactococcus reuteri* (log CFU/ml), glycerol concentration (mM), and incubation time (h) was set up and consisted of 18 experimental runs. The dependant factor was the relative reuterin concentration expressed through its antibacterial activity (Units reuterin per ml, U/ml) against *L. monocytogenes* using microbroth dilution assay. A polynomial equation for prediction of reuterin production (U/ml) produced by *L. reuteri* was created based on obtained data. Results showed that incubation time, bacterial and glycerol concentrations had linear positive effects on reuterin production. The quadratic effect of incubation time had negative effect on reuterin production in which at a fixed bacterial concentration of *L. reuteri*, a longer incubation time with low concentration of glycerol will cause the reduction in relative reuterin concentration. It was found that approximately 450 U/ml can be produced by *L. reuteri* with glycerol concentration ranging from 320 to 340 mM mM, *L. reuteri* concentration of 9.4 log CFU/ml and 2 h of incubation. Results demonstrated that applying suitable conditions for glycerol bioconversion into reuterin by *L. reuteri*, a high relative reuterin concentration could be obtained and used for food preservation against *L. monocytogenes*.

Keywords: Antibacterial activity, Reuterin, *Lactobacillus reuteri*, *Listeria monocytogenes,* response surface methodology

INTRODUCTION

Lactobacillus reuteri is a member of gastrointestinal bacteria of healthy human and animals. It has been demonstrated as a potential probiotic bacterium in biodetoxification and health stimulation, (**van Niel *et al.*, 2012**) and in immunomodulation (**Mechoud *et al.*, 2012**). Further, *L. reuteri* is well-known as a producer of 3-hydroxypropionaldehyde (3-HPA) or reuterin during anaerobic metabolism of glycerol (**Axelsson *et al.*, 1989; Chung *et al.*, 1989**). Glycerol is converted to reuterin by a coenzyme B_{12}-dependent dehydratase (**Talarico and Dobrogosz, 1990**). Reuterin can be further reduced by NADH to form 1,3-propanediol which is catalyzed by 1,3-propanediol: NAD oxidoreductase (**Talarico *et al.*, 1990**). Reuterin can inhibit the growth of yeast, protozoa, and many bacteria, both Gram-negative and Gram-positive (**Axelsson *et al.*, 1989; Chung *et al.*, 1989**).
Reuterin is water soluble compound, and it is active in a wide range of pH. It is also resistance to proteolytic and lipolytic enzymes (**El-Ziney *et al.*, 2000; Vollenweider and Lacroix 2004**). Therefore, potential of using reuterin for preservation of food products are of great interest (**El-Ziney and Debevere 1998; Arqués *et al.*, 2004; Montiel *et al.*,, 2014**). Reuterin could inhibit the growth of pathogenic bacteria such as *Listeria monocytogenes*, and *Escherichia coli* O157: H7 in milk and cottage cheese (El-Ziney and Debevere 1998), *L. monocytogenes* in cold-smoked salmon (**Montiel *et al.*, 2014**). A synergistic effect of reuterin plus nisin against *L. monocytogenes* and *Staphylococcus aureus* was obtained in refrigerated milk at the end of storage of 12 days (**Arqués *et al.*, 2004**).
It has been found that *L. monocytogenes* was more resistant to reuterin than some other bacteria. For example, reuterin at the same activity (8 U/ml) was more effective in eliminating *Staphylococcus aureus* than *L. monocytogenes* at 37 °C in milk (**Arqués *et al.*, 2004**). Similarly, it has been observed that *E. coli* strains were sensitive to reuterin than *Listeria* strains including *L. monocytogenes* (**El-Ziney *et al.*, 2000**). Thus, it is important to find suitable conditions so that *L. reuteri* can produce high reuterin concentration to eliminate *L. monocytogenes* in food products.
It has been demonstrated that several factors such as the glycerol and biomass concentrations, incubation time, temperature and pH can influence on reuterin production produced by *L. reuteri* (**Lüthi-Peng *et al.*, 2002a,b; Tobajas *et al.*, 2007**). However, most of studies focused on evaluation of varying effect of a single factor while other factors were fixed on reuterin activity and therefore, the possible interactive effects among different factors on reuterin production by *L. reuteri* could not be established.
Response surface methodology (RSM) is known as a combination of mathematical and statistical techniques for optimization of a process where dependent factors are affected by several independent factors. This method can be used to determine the possible interactive effects among different independent factors on the dependent factors in an experimental design (**Adjalle *et al.*, 2011; Khan *et al.*, 2014**).
Thus, the aim of this study was to evaluate the interactive effects of bioconversion condition on reuterin activity against *L. monocytogenes* using RSM. A central composite consisting of three independent factors (glycerol concentration, *L. reuteri* concentration and incubation time) at 5 levels was designed. The design formed 18 experimental bioconversion conditions. Reuterin production or relative reuterin concentration expressed through its activity (Units per ml, U/ml) against *L. monocytogenes* was determined using microbroth dilution method.

MATERIALS AND METHODS

Bacterial and growth conditions

Lactobacillus reuteri ATTCC 53608 was used in this study. The stock culture of *L. reuteri* in MRS broth containing 10 % glycerol was preserved as aliquot of 1 ml in cryovial tubes at -80 ºC. Before each experiment, one ml of stock culture was inoculated into test tube containing 9 ml of MRS broth, incubated at 37ºC for

24 h to obtain pre-inoculum. Then, the pre-inoculum (1.5 ml) was inoculated in Erlenmeyer containing 30 ml of MRS broth and incubated at 37 ºC for 24 h to obtain inoculum.

L. monocytogenes 2812 stock cultures were stored at -80 °C in Tryptic Soya Broth broth medium containing glycerol (10% v/v). Before each experiment, stock cultures were grown through two consecutive 24 h growth cycles in Tryptic Soya Broth at 37 °C. The cultivated cultures were centrifuged at 5000 g for 15 minutes and the obtained pellets were washed twice in sterile saline water (0.85% w/v) to obtain working cultures containing approximately 10^9 CFU/mL. The working cultures were diluted in sterile saline water (0.85 %, w/v) to have final concentration of 10^6 CFU/ml. This bacterial solution was then used for microbroth dilution assay to evaluate relative reuterin activity (U/ml).

Biomass production of *L. reuteri*

Preliminary experiment on the effect of fermentation time on reuterin production and on its antibacterial activity showed that biomass of *L. reuteri* collected at the end of exponential phase (24 h of fermentation) gave better antimicrobial activity against *L. monocytogenes*. Therefore, biomass of *L. reuteri* was collected at 24 h of fermentation.

The inoculum of *L. reuteri* was inoculated (5%, v/v) into Erlenmeyers containing 200 ml of MRS broth. The Erlenmeyers were incubated in an incubator at 37 ºC under static conditions for 24 h. The fermented broth was collected in centrifugal tubes and centrifuged at 5,000 x g for 10 min at 4ºC. The supernatant was discarded and biomass was washed twice with 50 mM sodium phosphate buffer (pH 7.5) to eliminate residual components of culture medium (MRS broth). The washing step was conducted by suspended biomass in 50 mM sodium phosphate buffer, then centrifuged at 5000 x g for 10 minutes at 4ºC and the supernatant was discarded after each washing step. Finally, biomass was suspended in 50 mM sodium phosphate buffer (pH 7.5). The cell concentration of *L. reuteri* (CFU/ml) in suspended biomass was enumerated by 10-fold serial dilution and plating on petri dishes containing MRS agar. Further, the suspended biomass was diluted by 1, 1.2, 1.4, 1.6, 1.8 and 2 folds and the optical density (OD) values of diluted solutions were recorded at 595 nm. Based on cell concentration determined by plating counting method and the OD of different diluted solutions of suspended biomass, the linear correlation between CFU/ml and OD values was established. This correlation helped to determine the approximately bacterial concentration of *L. reuteri* in subsequent experiments based on OD value of the cultures.

Experimental design for reuterin production

A central composite consisting of 3 independent factors (glycerol concentration, bacterial concentration and incubation time) at 5 levels (Table 1) was designed to find the suitable conditions for reuterin production against *L. monocytogenes*. This included 18 experimental bioconversion conditions (Table 2).

Table 1 Level of independent factors in the central composite design

Independent factors	Level				
	-2	-1	0	1	2
Glycerol concentration (X_1, mM)	166	200	250	300	334
Bacterial concentration (X_2, log CFU/ml)	8.0	8.3	8.7	9.1	9.40
Incubation time (X_3, h)	0.3	1	2	3	3.7

Bacterial suspension of *L. reuteri* at different concentrations was added into falcon tubes, and glycerol was added to obtain final concentration of bacteria and glycerol as required depending on each experimental condition (Table 2). The final volume of each falcon tube was 10 ml. The mixture in falcon tubes was mixed well and falcon tubes were anaerobically incubated at 37 ºC for different periods (h) as required in the experimental design. Then, tubes were centrifuged at 5 000 x g for 10 minutes and supernatant was collected. The supernatant was filtered through a 0.45 µm filter. The supernatant was kept at 4 ºC and used for evaluation of its activity against *L. monocytogenes* using microbroth dilution assay.

Table 2 Experimental runs and relative reuterin concentration

Experimental condition	Glycerol concentration (mM)	Bacterial concentration (log CFU/ml)	Incubation time (h)	Observed relative reuterin concentration (U/ml)	Predicted relative reuterin concentration (U/ml)
1	200	8.3	1.0	64.0	57.5
2	200	8.3	3.0	96.0	128.2
3	200	9.1	1.0	256.0	237.8
4	200	9.1	3.0	256.0	308.5
5	300	8.3	1.0	96.0	96.5
6	300	8.3	3.0	96.0	167.2
7	300	9.1	1.0	256.0	276.8
8	300	9.1	3.0	384.0	347.5
9	166	8.7	2.0	192.0	204.9
10	334	8.7	2.0	256.0	270.5
11	250	8.0	2.0	128.0	86.7
12	250	9.4	2.0	384.0	388.7
13	250	8.7	0.3	64.0	78.8
14	250	8.7	3.7	256.0	197.7
15	250	8.7	2.0	256.0	237.7
16	250	8.7	2.0	256.0	237.7
17	250	8.7	2.0	256.0	237.7
18	250	8.7	2.0	256.0	237.7

Evaluation of reuterin production (U/ml) using microbroth dilution assay

Reuterin production was evaluated by relative reuterin concentration which is expressed through its antibacterial activity (U/ml) using dilution assay (**Chung *et al.*, 1989**). In this study, *L. monocytogenes* was used as target bacterial strain and the microbroth dilution assay with some modification was applied for evaluation of reuterin production (**Turgis *et al.*, 2012**). The supernatants obtained from experimental conditions were diluted by 8, 12, 16, 24, 48, 96, 128 and 192 times using sterile sodium phosphate buffer (50 mM, pH 7.5). First, 125 µl of sterile Mueller-Hinton Broth (MHB) were filled into wells of a microplate. Then, 50 µl of reuterin solution at different concentrations were added into wells of eight columns of the microplate. Finally, 25 µl of working culture of *L. monocytogenes* (10^6 CFU/ml) were inoculated into the wells of six rows of the microplate. The last two rows containing MHB, different concentrations of reuterin and without *L. monocytogenes* served as blank. The columns containing MHB with *L. monocytogenes* and without reuterin served as positive control. The final volume of the wells in the microplate was 200 microliters. Thus, the final reuterin concentration in the tested wells was diluted by 4 fold. This factor plus the dilution level mentioned above (8, 12, 16, 24, 48, 96, 128 and 192) were considered in calculation the reuterin activity against *L. monocytogenes*. The microplate was incubated for 24 h at 37 ºC, then, the optical absorbance (OD) of the microplate was measured at 595 nm using an Ultra Microplate Reader (Biotek instruments, Winooski, VT, USA). Relative reuterin concentration (Unit reuterin per ml or U/ml) was calculated as the reciprocal of the highest dilution of the sample that can inhibit the growth of *L. monocytogenes* (**Chung *et al.*, 1989**).

Statistical analysis

Data obtained from experimental design were used for analysis of variance (ANOVA), and regression analysis using STATISTICA 12 (STATSOFT Inc., Tulsa, US). A general second-order polynomial equation was built to predict reuterin production (equation 1).

$$Y = B_0 + \sum_{i=1}^{3} B_i X_i + \sum_{i=1}^{3} B_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} B_{ij} X_i X_j \quad \text{(equation 1)}$$

where Y is the predicted response; B_0, intercept; X_i and X_j are values of various levels of the independent variables; B_i is the values of linear coefficients; and B_{ii} is the values of quadratic coefficients and Bij is the values of the interaction between 2 independent factors (Adjallé et al., *2011*). Response surface plots were created to show the effects between the independent variables (glycerol concentration (X_1), bacterial concentration (X_2) and incubation time (X_3)) on the reuterin production (U/ml).

RESULTS

Analysis of variance for reuterin production and regression coefficients of factors for the model

The relative reuterin concentrations (U/ml) of 18 experimental bioconversion conditions are presented in Table 2. It can be observed that depending on conditions of three independent factors (glycerol, bacterial concentrations and incubation time), the observed relative reuterin concentration could vary from minimum of 64 to maximum of 384 U/ml. The higher relative reuterin concentration (U/ml) of a sample means that even at higher factor of dilution of this sample, it can still have the capacity to eliminate *L. monocytogenes* in tested system.

Analysis of variance (ANOVA) was conducted and the model had a regression coefficient factor (R^2) of 0.89, indicating that 89% of the variation in the responses could be explained by the combination of the responses. Further, there was no lack of fit of the model. Thus, the model can be used for prediction of reuterin production based on incubation time, glycerol, and bacterial concentration.

The reduced ANOVA table (after eliminating the factors which are not important in the model) is presented in Table 3. It can be observed that the linear effect of bacterial concentration was a very important factor on reuterin production (P = 0.000001). The linear and quadratic effects of incubation time were also important to affect reuterin production (P = 0.004). The linear effects of glycerol (P = 0.07) was important on reuterin production but less than those of incubation time and bacterial concentration (Table 3).

Table 3 Analysis of variance for reuterin production

Source	Sum of square	df	*P* value
Glycerol concentration (L)*	5244.9	1	0.07
Bacterial concentration (L)	110631.7	1	0.000001
Incubation time (L)	17075.4	1	0.004
Incubation time (Q)	16846.3	1	0.004
Total sum of square	167876.5		

* L and Q represent for linear and quadratic effect. There is no lack of fit of the model.

Table 4 Regression coefficients of the predictive model

Factors	Regression Coefficient	*P* value
Constant	-2032.00	0.000001
Glycerol concentration (L)*	0.39	0.07
Bacterial concentration (L)	225.39	0.000001
Incubation time (L)	175.94	0.0009
Incubation time (Q)	-35.15	0.004

* L means Linear effect, Q means Quadratic effect.

The final regression coefficients of the factors for the model are presented in Table 4 and were used to create a predictive model (equation 2):

$Y = -2032.00 + 0.39X_1 + 225.39X_2 + 175.94X_3 - 35.15X_3^2$ (equation 2)

where Y = reuterin activity (U/ml); X_1 is glycerol concentration (mM); X_2 is *L. reuteri* concentration (log CFU/ml); and X_3 is incubation time (h).

It can be observed that all linear effect of glycerol, biomass and incubation time have positive effects on the reuterin production while the quadratic effect of incubation time have a negative impact on the activity (Table 3). Based on the model, the predicted values of relative reuterin concentration (U/ml) of experimental runs are also presented in the Table 2.

Response surface plots of the interactive effects between different independent factors on reuterin activity

Response surface plots of the relation between different independent factors on the reuterin production was created and expressed in Figure 1 to Figure 3.

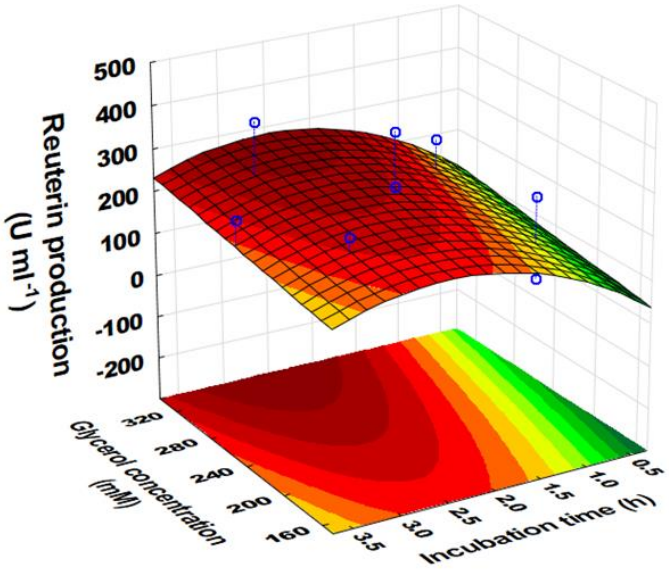

Figure 1 Effect of glycerol concentration and incubation time on reuterin production (bacterial concentration was fixed at 8.7 log CFU/ml)

Figure 1 presents the relation between glycerol concentration (X_1, mM) and incubation time (X_3, h) on reuterin production when the concentration of *L. reuteri* (X_2) was kept constant at 8.7 log CFU/ml. It can be observed that increase in both incubation time (from 0.5 to about 2.5 h) and glycerol concentration (140-340 mM) can cause an increase in relative reuterin concentration; however, further increase in incubation time and at low glycerol concentration, a decrease in relative reuterin concentration can be occurred. For example, when the incubation time is around 1 h, and glycerol concentration is from 140 to approximately 200 mM, a relative reuterin concentration of equal or less than 140 U/ml can be produced by 8.7 log CFU/ml of *L. reuteri* (Figure 1). When glycerol concentration is from 280 to 340 mM and the incubation time is from 2 to 3 h, almost 300 U/ml can be produced by the same concentration of *L. reuteri* (8.7 log CFU/ml). However, when the incubation time is more than 3 h and glycerol concentration is less than 240 mM, relative reuterin concentration of less than 200 U/ml can be obtained (Figure 1). Thus with a fixed bacterial concentration of *L. reuteri*, a longer incubation time with low concentration of glycerol will cause the reduction in reuterin production.

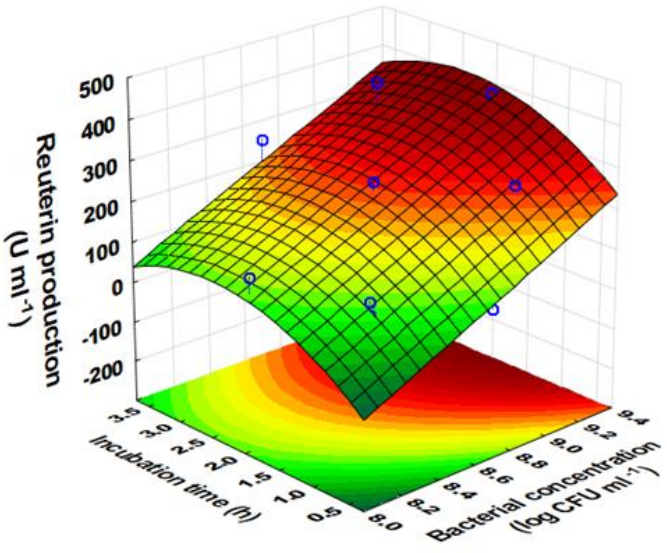

Figure 2 Effect of bacterial concentration and incubation time on reuterin production (glycerol concentration was fixed at 250mM)

Figure 2 presents the relation between bacterial concentration (X_2, log CFU/ml) and incubation time (X_3, h) on reuterin production when glycerol concentration was kept constant at 250 mM. In general, it can be observed that the increase in bacterial concentration (from 8 to 9.4 log CFU/ml) and the increase in incubation time (from 0.5 to 2.5 h) cause a significant increase in relative reuterin concentration; however, further increase in incubation time (more than 2.5 h) of solution containing 250 mM glycerol with irrespective of bacterial concentrations

cause a decrease in reuterin production (Figure 2). It can be seen that high relative reuterin concentration (approximately 400 U/ml) can be obtained using 9.4 log CFU/ml of *L. reuteri*, 250 mM glycerol and 2.5 h of incubation. With the same conditions but with the incubation time of 3.5 h, only 350 U/ml against *L. monocytogenes* can be obtained.

Figure 3 presents the interactive effects between glycerol concentration (X_1, mM) and bacterial concentration (X_2, log CFU/ml) on reuterin production when the incubation time was kept constant at 2h. It can be observed that increasing both *L. reuteri* and glycerol concentrations cause an increase in reuterin production. It is of interest to observe that since the incubation time is fixed, a lower concentration of glycerol require a higher concentration of bacteria or a higher concentration of glycerol require a lower concentration of bacteria to produce similar reuterin concentration (similar antilisterial activity). For example, when glycerol concentration is around 200 mM, and bacterial concentration is around 8.8 log CFU/ml, a relative reuterin concentration of approximately 200 U/ml can be obtained. Similarly, when glycerol concentration is around 280 mM, and bacterial concentration is around 8.6 log CFU/ml, a relative reuterin concentration of approximately 200 U/ml can also be obtained. With incubation time of 2 h, a higher relative reuterin concentration of approximately 450 U/ml can be obtained by using the bacterial concentration of approx. 9.4 log and a glycerol concentration of 320 to 340 mM (Figure 3). Figure 3 also showed that further increasing glycerol and bacterial concentration at fixed incubation time of 2 h may obtain higher relative reuterin concentration.

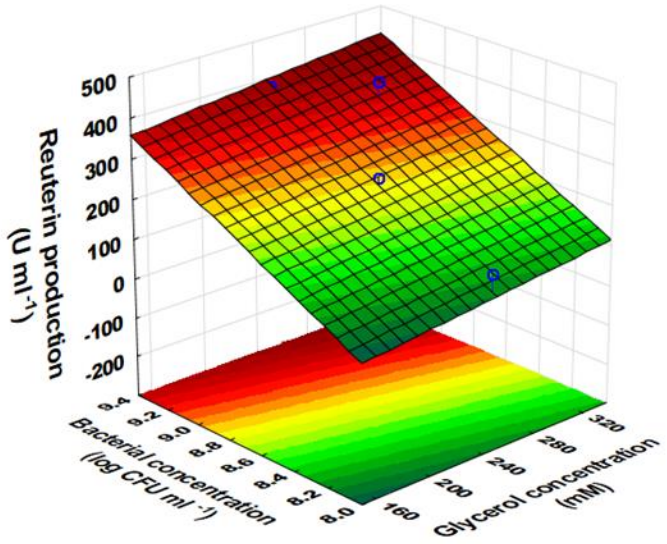

Figure 3 Effect of glycerol concentration and *L. reuteri* concentration on reuterin production (incubation time was fixed at 2 h)

DISCUSSION

Reuterin (3-hydroxypropionaldehyde, 3HPA) is a low-molecule-weight, nonproteinaceous antimicrobial substance containing aldehyde group. Reuterin is not a bacteriocin (**Vollenweider and Lacroix 2004**) which is different with other proteinaceous bacteriocins which are produced by many other strains of lactic acid bacteria (LAB) **Turgis *et al.*, 2013**). In addition to *L. reuteri*, some other bacterial genera such as *Klebsiela*, *Enterobacter*, *Citrobacter*, *Clostridium*, and *Bacillus* can also produce reuterin (**Vollenweider and Lacroix 2004**).
It has been observed that *E. coli* strains were sensitive to reuterin than *Listeria* strains including *L. monocytogenes* (**El-Ziney *et al.*, 2000**). **El-Ziney and Debevere (1998)** found that adding reuterin at 100 and 150 Units g^{-1} cheese caused a reduction of *E. coli* by 3 and 6 log CFU, respectively, while a reduction of 2 and 5 log was observed for *L. monocytogenes* at day 7 of storage. The results showed that *L. monocytogenes* was more resistant to reuterin than that of *E. coli*. Thus, to apply reuterin as an antimicrobial agent against foodborne pathogens such as *L. monocytogenes*, a high reuterin concentration is required. Therefore, current study focused on effects of different combined conditions (Table 2) on bioconversion glycerol into reuterin by *L. reuteri* and the relative reuterin concentration was evaluated through its activity against *L. monocytogenes*.
It was found that incubation time, bacterial and glycerol concentration had positive effect on reuterin production. It showed that utilization of 320-340 mM glycerol, 9.4 log CFU/ml of *L. reuteri* and incubation time of 2 h, a high relative reuterin concentration of approximately 450 U/ml can be obtained (Figure 3). However, at a fixed concentration of bacterial and glycerol concentrations, longer incubation time caused a decrease in reuterin production. The decrease in relative reuterin concentration or its activity after long incubation time was also observed by other studies (**Lüthi-Peng *et al.*, 2002b**). The decrease in its activity could be due to reuterin was reduced to form 1,3-propanediol (**Slininger *et al.*, 1983; Talarico *et al.*, 1990; Vollenweider and Lacroix, 2004**). Further, it is also known that reuterin at high concentration can also be toxic for *L. reuteri*, the producer (**Lüthi-Peng *et al.*, 2002b; Vollenweider and Lacroix, 2004**).
Concerning the mechanism of action of reuterin, it was proposed that the aldehyde group of reuterin is mainly responsible for antimicrobial activity (**Schaefer *et al.*, 2010; Vollenweider *et al.*, 2010**). **Schaefer *et al.*, (2010)** demonstrated that reuterin induce oxidative stress in target organisms. The authors confirmed that the aldehyde form interacting with thiol groups of small molecules and proteins of target organisms and cause growth inhibition. When cysteine was added into growth media of *E. coli* or *Clostridium difficile* before exposing to reuterin, the antibacterial effects of reuterin against these bacteria was suppressed (**Schaefer *et al.*, 2010**). In other study, **Vollenweider *et al.*, (2010)** demonstrated that reuterin induces a depletion of reduced glutathione (GSH) in *E. coli* and then attack on protein- linked SH- groups, changing protein functions which finally result in cell death. Further, adding exogenous GSH caused an increase in intracellular GSH concentrations up to 9-fold and prevented the alteration of protein thiols, and resulted in rescuing the bacteria (**Vollenweider *et al.*, 2010**). These properties explain for the reason why reuterin can act against a broad spectrum of bacteria, yeast, protozoa and it is a candidate for food preservation.

CONCLUSIONS

This study evaluated the bioconversion conditions including incubation time, glycerol and concentration of *L. reuteri* on reuterin production using response surface methodology and the relative reuterin concentration was expressed through its activity against *L. monocytogenes*. A polynomial predictive equation for relative reuterin concentration produced by *L. reuteri* was established. Incubation time, glycerol and *L. reuteri* concentrations had positive effects on reuterin production. At a fixed concentration of bacteria, longer incubation time and low concentration of glycerol caused a decrease in reuterin production. It was found that with glycerol concentration of 320-340 mM, 9.4 log CFU/ml of *L. reuteri* and 2 h of incubation, a high relative reuterin concentration of approximately 450 U/ml against *L. monocytogenes* can be obtained. Future study on application of reuterin alone or in combination with other antibacterial agents such as essential oils, organic acid salts to control *L. monocytogenes* or other foodborne pathogens in food products is necessary.

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BIOSCOURING OF WOOL USING PROTEASE FROM *BACILLUS SUBTILIS* ISOLATED FROM ABATTOIR WASTE

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ABSTRACT

Bioscouring refers to the enzymatic removal of impurities from fibres/fabrics, which endows it with improved hydrophilicity for further wet processes. Enzymatic scouring preserves the fibre's structure and strength, avoids high energy consumption and severe pollution problems that are associated with conventional alkaline treatments. In the present study, protease enzyme was extracted from *Bacillus subtilis* which was isolated from abattoir (slaughter house) waste. Different medium parameters were optimized for maximal enzyme production. The enzyme was partially purified using ammonium sulphate precipitation followed by dialysis bag method. Partially purified protease enzyme was used in the bioscouring of wool fibres. Different parameters such as pH, temperature, time, enzyme concentration were optimized to achieve an efficient scouring. Comparison of enzymatic process for wool fibre with conventional alkaline soap process in terms of weight loss, whiteness index, tensile strength and FTIR studies confirmed that bioscouring could be as effective as the conventional process.

Keywords: Bioscouring, Protease, *Bacillus subtilis*, Abattoir waste, Wettability, Alkali solubility, Whiteness index

INTRODUCTION

Wool is a natural protein fibre which is obtained from sheep skin. Raw wool fibers obtained from the sheep contain greasy substances referred to as impurities, as high as 40-50 %. Sabaceous glands of sheep's skin secretes oils and fats, often referred to as wool fat/grease, essentially a mixture of higher fatty alcohols and acids **(Saravanan *et al*., 2014)**. The contaminants consist of 5-25% grease, 2-15% suint and 5-20% dirt, moisture, vegetable matter, sand and dirt. All these impurities tend to make the raw wool highly hydrophobic; which has to be removed to make it hydrophilic prior to its further processing. However, entire removal of wool wax is not desirable at fibre stage, in order to facilitate the lubricating actions among the fibers during subsequent spinning processes. The conventional methods of wool scouring utilize high amount of chemicals, detergent, alkali etc which create some serious problems; both, for the environment and the industry while effluent treatment and disposal **(Halliday, 2002)**.

Traditionally, wool scouring is carried out by using alkali and soap at temperature 60ºC - 80ºC. The alkaline scouring treatment emulsifies the waxes and breaks down peptide bonds into water-soluble or water-emulsifiable products that are later washed off from the wool materials. This process effectively removes all impurities that exist in the raw wool fibres but has a high energy requirement and the effluent is ecologically undesirable because of its high alkalinity, biochemical and chemical oxygen demand. These drawbacks in the process led to a consideration of alternatives. Bioscouring of wool with suitable enzymes appears to be most promising in this respect. The necessity to use more environmental friendly processes leads to the replacement of conventional chemicals by enzymatic ones. Bioscouring is a novel process based on the idea of particularly targeting fats, waxes, suints, dirt etc. with specific enzymes.

Zheng et. al have reported a method to optimize the wool-scouring process with bio-enzymes of *Bacillus Subtilis* and *Candida lipolytica*. **(Zheng et. al., 2012).** Enzymatic treatment of textiles has been of great interest because of its effectiveness under mild treatment conditions **(Cardamone *et al*., 2006)**. Enzymes act in the pH range between 5 and 8, at temperatures around 30 to 40ºC at atmospheric pressure while conventional method requires pH 10.8 at temperature around 60ºC - 80ºC. These treatments also enhance many textile properties such as wettability, dye uptake and polymer adhesion **(Negri *et al*., 1993; Brack *et al*., 1999)**.

From an environmental point of view, enzymes are active in small doses and highly biodegradable; hence, the use of enzymes in scouring helps in reduction of the auxiliary agents, which usually are poorly biodegradable.

As per the literature, various enzymes like protease, lipase, pectinase and amylase have been used to carry out treatments on wool **(Saravanan *et al*., 2014; Sayad *et al*., 2010; Hmidet *et al*., 2009)**.

Proteases or proteinases are proteolytic enzymes which catalyze the hydrolysis of proteins. Based upon their structures or properties of the active site, there are several kinds of proteases such as serine, metallo, carboxyl, acidic, neutral and alkaline proteases. Proteases are industrially important enzymes and constitute a quarter of the total global enzyme production **(Kalaiarasi and Sunitha, 2009)**. Proteases are industrially important due to their wide applications in leather processing, detergent industry, food industries, pharmaceutical, textile industry etc **(Deng *et al*., 2010; Jellouli *et al*., 2009)**.

Commercially in wool industry, protease enzyme is used due to its substrate specificity. This enzyme tends to remove desired impurities like wax, suint, sand and vegetable matter etc. from raw wool fibre to make it hydrophilic. Effective removal of wax from raw wool substrate with the enzyme under mild conditions will provide high quality of wool for the subsequent dyeing and finishing processes with less energy consumption under safer conditions. In contrast to drastic alkaline conditions conventionally used, treatment with protein degrading enzymes would not affect the internal structure of wool fibre and thus avoid fibre damage. Wide scale industrial application of protease requires their cost effective production to make the process economically viable **(Shukla, 2001; Karmarkar 1999)**.

Pre-treatment of wool fiber with enzyme leads to increase its hydrophilicity with enhanced swelling properties. Protease can catalyse the degradation of different component of wool fibre **(Hooda 2013)**. Protease enzyme penetrates into amorphous region and causes swelling and it leads to changes in the disulphide region of cystine than amide components during chemical degradation. **((L. Ammayappan 2013)**.

In the present investigation, a successful attempt has been made for production of protease using submerged fermentation (SmF) from a newly isolated strain of *Bacillus subtilis* from degraded slaughter house waste material and its application in the bioscouring of wool fibre in textile industry. The parameters essential for effective wool scouring like time, temperature, pH, enzyme concentration, etc were optimized.

MATERIAL AND METHODS

Raw Materials

The raw greasy merino wool fibres of Australian origin were procured from (WRA) Wool Research Association, Thane, Mumbai, India for the scouring experiments being difficult to scour as they contain the high amount of contaminants such as wool wax, dirt, dust and suint. Protease enzyme used for bioscouring was extracted from *B. subtilis* obtained from abattoir waste at our laboratory. Identification of the bacteria was carried out by 16S rRNA sequencing method. Casein was purchased from Sigma Chemicals. Chemicals for microbiological experiments were supplied by Himedia, Mumbai and all other chemicals were procured from S.D. Fine chemicals, Mumbai.

Microorganism and enzyme production

The *B. subtilis* used in this study was isolated from degraded abattoir (slaughter house waste), collected from local market, Mumbai, India. For production of protease enzyme, 100 ml of nutrient medium containing 1% gelatin w/v (as protein source), 0.5% yeast extract and 100 ml distilled water at pH 14 was inoculated with 1% inoculums of 24 h old *B. subtilis* culture (1×10^8 CFU/ ml). As reported earlier, *Bacillus species* has optimum temperature of 40ºC for its growth and its matabolic activity is at highest peak during its growth phase due to which the enzyme works efficently **(Badhe et. al 2016)**. The flasks were incubated at 40º C for 24 h on a rotary shaker maintained at 150 rpm. After 24 h the broth was centrifuged at 5000 rpm at 4º C for 20 min and the cell free supernatant was collected. The supernatant was used as crude enzyme extract. The application of enzyme in the textile industry does not require high grade purity for enzymes and generally requires use of the crude or the partially purified enzyme preparation **(Nerurkar et. al 2013)**. Thus, partial purification of the protease was carried out by ammonium sulphate precipitation since ammonium sulphate is highly soluble in water, cheap, and has no deleterious effect on the structure of protein. Ammonium sulphate was added to the crude enzyme sample to get 30% saturation and was kept overnight at 4ºC. It was then centrifuged at 6000 rpm for 20 min. The precipitate was suspended in phosphate buffer, pH 7.0 and was dialyzed against the same buffer to carry out desalting process.

Protease assay

Protease activity was measured by caseinolytic method, **(Walter, 1984)**. One unit of enzyme was defined as the amount of enzyme that liberates peptide fragments equivalent to 1 mg of bovine serum albumin (BSA) under the assay conditions.

Conventional scouring

Conventional scouring was carried out at pH 10 for 2 h at 35ºC to 40ºC. The solution used for scouring contained 2 g/L Auxipon NP solution (non-ionic surfactant) and 3 g/L sodium carbonate solution (alkali). MLR was kept at 1:30. Sample was given hot wash. The fibres were firstly air dried and then conditioned at 20 ± l ºC and 65 ± 2% RH for at least 24 h in desiccator containing calcium chloride.

Bioscouring

Different parameters of bioscouring namely buffer pH, enzyme concentration, temperature and time period were optimized to examine the efficiency of protease from *B.subtilis* and to further obtain efficient removal of impurities. The raw wool fibres were subjected to four different treatment solutions stated below:

1. Phosphate buffer at pH 7.0 and enzyme (B+E)
2. Phosphate buffer, soap and enzyme(B+S+E)
3. Phosphate buffer, alkali and enzyme (B+A+E)
4. Phosphate buffer, soap, alkali and enzyme (B+S+A+E)

The raw wool fibre samples were subsequently suspended in buffer of pH ranging from 3.0 to 11.0. The material to liquor ratio was set at 1:30 with varying dosage of protease in the range of 2% to 14% on the weight of fabric for treatment time of 30, 60, 90, 120, 150 and 180 min at different temperatures ranging from 30 ºC to 80 ºC. The bioscouring process optimization was carried out by varying one parameter at a time, keeping other factors constant. The experiments were performed in the water bath (Bio Technics India). After the enzymatic treatment; the fibres were given hot wash at boiling temperature for 30 min, air dried and dried in desiccator for 24 h.
Efficiency of bioscouring for each parameter was evaluated by monitoring weight loss of the fibres as the difference before and after the bioscouring treatment. Wool sample, bioscoured using protease enzyme under optimized parameters, was used for further analysis.

Evaluation of fibre properties

Weight loss determination

Enzyme-treated and untreated wool fibers were conditioned at 20 ± l ºC and 65 ± 2% RH for at least 24 h before being weighed. Weight loss was expressed as:

$$\text{Weight loss } \% = \frac{W1-W2}{W1} \times 100$$

Where, W_1 is the conditioned weight of the sample before enzymatic treatment, and W2 is the conditioned weight of the sample after enzymatic treatment respectively.

Wettability (Drop test)

The wettability of raw wool and enzyme treated wool fibres was measured using standard test, BS 4554:1970. According to the method, the time (sec) required by a drop of water to be absorbed by the fibre is defined as the ability of the fibre to get wet.

Sinking time

Sinking time test AATCC 17-2005 was modified for being used for fibre. Instead of fabric, a bundle of fires was used as indicative test for evaluating absorbency.

Alkali solubility

To quantify the damage to the epicuticle of wool, alkali solubility in terms of weight loss of raw wool and enzyme treated fibres was measured using IWTOTM-4-00 standard test method. The values were calculated as a percentage of the original mass, according to the equation given below,

$$\text{Alkali solubility } (\%) = \frac{M1 - M2}{M1} \text{ X } 100$$

Where, M1 is the mass of oven dry sample before sodium hydroxide treatment, and M2 is the mass of oven dry sample after sodium hydroxide treatment.

Residual grease content

Efficiency of removal of grease content of conventional scoured and enzyme scoured wool sample was evaluated by using the following formula according to standard method IWTO1903:

$$\text{Grease Content} = \frac{W1 - W2}{W1} \times 100$$

Where, W1 is the weight before removal of grease from wool sample and W2 is the weight after removal of grease from wool sample.

Determination of Moisture Regain

Moisture regain was calculated using the following equation according to ASTM method 2654-76:

$$\text{Moisture regain } (\%) = \frac{W1 - W2}{W2} \times 100$$

Where, W1 is the weight of the conditioned sample at standard humidity and W2 is the weight of the sample dried to constant weight.

Determination of Moisture Content

The samples were preconditioned in a desiccator for 24 hr at 65 ± 2 % RH and 27 ± 2ºC. The moisture content was determined after obtaining the weight of wool dried at 105ºC for 3 hr. The oven dry mass was determined according to standard IWTO-34-85-E method.

$$\text{Moisture Content } (\%) = \frac{W1 - W2}{W1} \times 100$$

Where, W_1 is the weight of the conditioned sample at standard RT and temperature and W_2 is the weight of the sample dried to constant weight.

Single Fiber Strength Test

The single fiber strength of raw and scoured wool was measured on Universal Tensile Machine supplied by Aimil Limited, Navi Mumbai according to ASTM D 3822 standard test method. The instrument was based on constant rate of

elongation (CRE) principle. The distance between jaws was 10mm and the travel rate was 6 mm/min.

Fiber Mean Diameter Test

Fiber diameter measurement was carried out using OFDA 100 as per the standard IWTO472011. The fiber samples were cut into 2 mm snippets and spread on a 70 mm square glass slide. The whole slide was scanned with a minimum of 6000 fibers. For each sample, three measurements were taken. The mean diameter and standard deviation of the sample were then calculated.

Whiteness & Yellowness measurement

The ASTM whiteness Index (WI) and yellowness Index (YI) of samples, before and after scouring were determined by using Computer Colour Matching (CCM) System (Spectrascan5100+) according to IWTO3503 standard test method. The Improvement in whiteness and reduction in yellowness are expressed as the percentage change relatively to the original whiteness and yellowness respectively.

FTIR analysis

FTIR study was carried out to analyze the changes in structural groups and impurities on the wool surface of raw wool and enzyme treated wool fibres.

SEM

The surface morphologies of the wool samples were visualized using a JSM-6380L, an analytical scanning electron microscope (JEOL Company, Japan), operated at a typical accelerating voltage of 5 kV. The samples were sputter-coated with Platinum for 30 s at 15 mA prior to the observation.

Statistical Analysis

All the experiments were conducted in triplicate and results were expressed as mean±standard deviation. Student's t-test was used to analyze data, and statistical significance was declared at $p < 0.05$.

RESULTS AND DISCUSSION

Protease production in submerged fermentation

Glucose was used as sole carbon source for protease production using B. *subtilis* in submerged fermentation. Gelatin was used as protein source as a substrate. Protease so produced showed maximum protease activity of 63 U/ml after optimizing various physical, chemical parameters of fermentation and partial purification using Ammonium sulphate.

Bioscouring

Raw wool fibres were subjected to four different treatment solutions as reported above. It was seen from (Figure.1) that there was maximum weight loss and a less than 2 sec wetting time when raw wool was treated with the solution containing buffer and enzyme (B+E). Solution B+E was found to be optimum and was used for further studies, due to that protease enzyme is stable in buffer solution **(Joshi et al. 2013).**

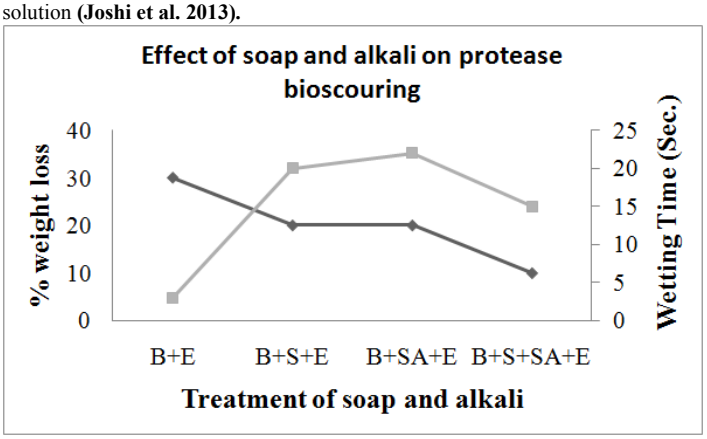

Figure 1 Effect of four different treatment solutions on protease bioscouring.
Protease scouring was carried out at protease dose of 2 % (owf), pH 7.0, at 40ºC keeping MLR at 1:30. Filled squares indicate percentage weight loss of the wool fibre and filled diamonds indicate wetting time of wool fibres in second.
Four different treatment solutions: solution B+E containing 50 mM phosphate buffer and enzyme, solution B+S+ E containing buffer, soap and enzyme, solution B+A+ E containing buffer, alkali and enzyme, solution B+S+A+ E containing buffer, soap, alkali and enzyme.

In order to explore the potential of our protease enzyme in bioscouring of wool, various factors like pH, enzyme concentration, temperature and time period were optimized. As seen from (Figures. 2–5), the optimal conditions for the bioscouring of wool sample were 4% protease dosage on the weight of the fibre at 60 ºC, Phosphate buffer pH 7.0 and a treatment time of 120 min.

Effect of Time period

Effective bioscouring using protease was optimized for 120 min in terms of weight loss and wettability. From the (Figure.2), it can be seen that the weight loss remained steady upto 60 min, after that there was a steep rise and the maximum weight loss was observed at 120 min. After that there was a sudden decline in weight loss which can be attributed to protein specific action of protease such that within treatment time of 120 min itself, protein and hence the other loosened impurities get removed.

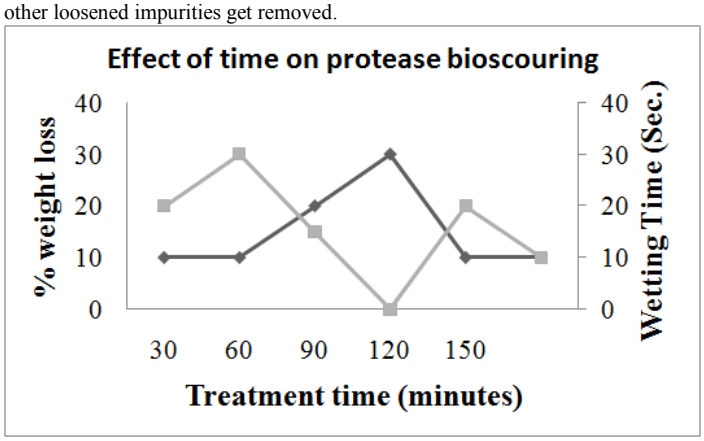

Figure 2 Effect of time period on protease bioscouring.
Protease scouring was carried out at protease dose of 2 % (owf), pH 7.0, at 40ºC for varying period of time keeping MLR at 1:30. Filled squares indicate percentage weight loss of the wool fibre and filled diamonds indicate wetting time of wool fibres in second.

Effect of temperature

Effect of temperature on protease scouring can be seen from (Figure. 3). As compared to 20% weight loss obtained in alkaline scouring, sufficient weight loss is achieved when scouring is carried out between temperature ranges of 30ºC and 80ºC, where as scouring efficiency decreases drastically as temperature of scouring bath is raised to 80ºC. This is because the protease enzyme must be showing less activity at 80ºC as compared to lower temperatures mentioned earlier. Optimum temperature for scouring of wool using protease was observed as 60ºC.

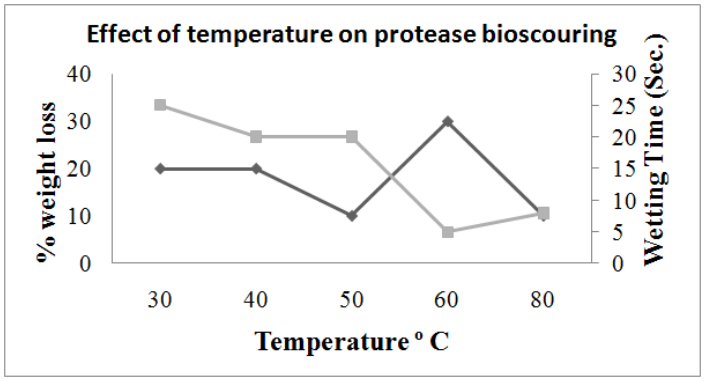

Figure 3 Effect of temperature on protease bioscouring.
Protease scouring was carried out at protease dose of 10% (owf), pH 7.0 for 120min at various temperatures keeping MLR at 1:30. Filled squares indicate percentage weight loss of the wool fibre and filled diamonds indicate wetting time of wool fibres in second.

Effect of enzyme concentration

As can be seen from (Figure. 4), 4 % of protease enzyme (owf) is optimum for scouring as further increase in the protease concentration showed more or less similar weight loss as compared to weight loss achieved when 4% of protease enzyme was used for scouring.

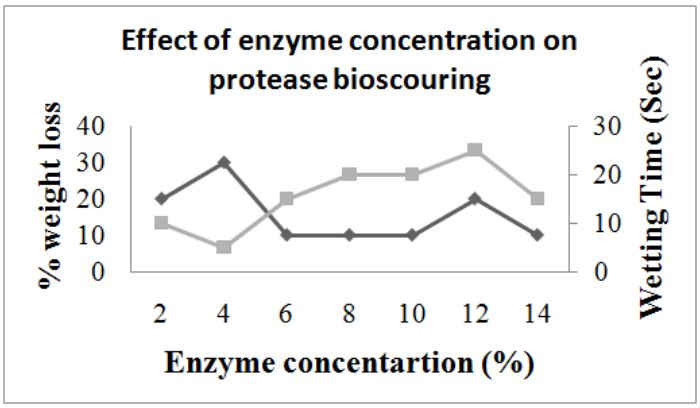

Figure 4 Effect enzyme concentration on protease bioscouring.
Protease scouring was carried out at pH 7.0, 40ºC for 120 min at various concentration of the protease keeping MLR at 1:30. Filled squares indicate percentage weight loss of the wool fibre and filled diamonds indicate wetting time of wool fibres in second.

Effect of pH

As can be observed from (Figure.5), pH of scouring bath greatly affects scouring action of the protease enzyme. Optimum pH for scouring of wool using protease was observed as 7.0.

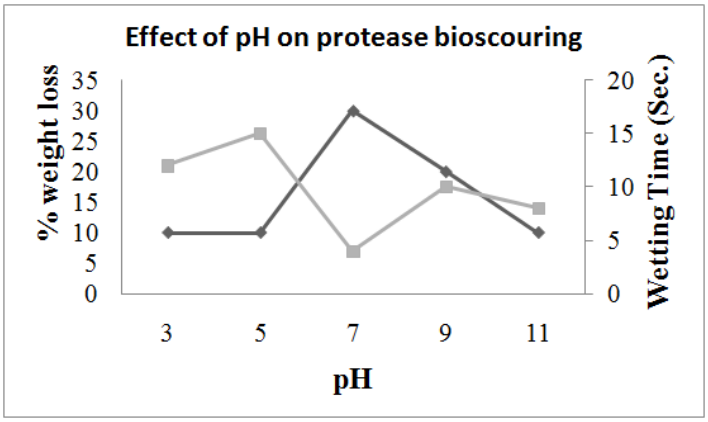

Figure 5 Effect of pH on protease bioscouring.
Protease scouring was carried out at protease dose of 10% (owf), pH 7.0 for 120 min at 60ºC for varying pH, keeping MLR at 1:30. Filled squares indicate percentage weight loss of the wool fibre and filled diamonds indicate wetting time of wool fibres in second.

Wettability of fibre

The raw wool fibers were tested for wetting time after it was subjected to scouring. The comparison of wetting time for B+E solution, conventional i.e. B+S+A+E solution and raw wool fibers. It was found that after treating raw wool with B+E solution, there is increase in its hydrophilicity and wettability was found to be less than 2 s. The raw wool fibres were not able to absorb the water droplet; it was observed that, after more than 30 min also the water drops remain as it is and can be rolled off easily from the fiber surface without wetting the surface.

Sinking time

Sinking time of both, conventionally scoured and protease scoured wool sample was found to be less than 2 s. Optimum temperature for bioscouring was found to be 60ºC. At this temperature, though some of the fats or waxes from wool fibre get loosened up, it still adheres to the fibre surface. When hot wash is given, they leach out completely in the bath; allowing the fibre to sink analogous to alkaline scoured fibre.

Alkali solubility

The alkali solubility of the enzyme treated fibers was tested and compared with the raw wool fibres. The alkali solubility values for wool fibres reported in literature are between 9% and 15% for undamaged wool **(Atav and Yurdakul, 2011)**. The alkali solubility for raw wool and enzyme treated wool was found to be 9.10% and 11.48%.

Residual grease content

Efficiency of removal of grease content of raw wool which was 11.90% for conventional scoured, (B+S+A+E) scoured was 0.16% and enzyme scoured (B+E) wool sample was 0.36 %.

Moisture Regain and moisture content

Removal of hydrophobic greasy substances from the surfaces of the raw wool is expected to increase the moisture regain values and higher weight loss in the scouring treatment translates to higher moisture regain values. The highest moisture regain values were observed in the case of enzyme scoured i.e. B+E samples at 42.85 %, while the moisture regain values of conventional i.e. B+S+A+E scoured, B+S+E scoured samples, or B+A+E scoured wool samples were in the range of 25 %. According to standard method, enzyme and buffer showed maximum moisture regain and content as compared to other.

Effect on Physical properties

Fiber Mean Diameter Test and Single Fiber Strength Test

There was no significant effect on mechanical properties of wool fibers on bioscouring. Little difference in the tenacity and elongation of wool fibers is attributing of high degree of variability in fiber dimension and non uniformity in wool. It has also been found that there is no difference in fiber diameter of all the samples as shown in (Table. 1). The fiber diameter ranges between 19.2-19.5 micron (Table. 2) **(Kalantzi et al., 2008)**.

Table 1 Effect of Protease and alkaline scouring on tensile strength of wool fibers

Serial no.	Sample	Breaking strength	Extension (%)
1	Raw wool	5.80	27
2	Alkaline scouring (B+S+A+E)	6.00	37
3	Protease Scouring (B+E)	4.10	38
4	B+ S+ E	5.30	34
5	B+A+E	4.80	35
6	B+S+A+E	4.10	50

Table 2 Effect of protease scouring on the properties of wool fiber.

Sr. no	Fibre sample	Raw wool	Alkaline scoured (B+A+S+E)	Protease Scoured (B+E)	(B+S+E)	(B+A+E)	(B+S+A-
1	Average fibre fineness (micron) (IWTO 47)	19.3	19.2	19.2	19.2	19.2	19.2
2	SD	1.13	1.27	1.59	1.11	1.33	1.46
3	CV%	21	31	27	26	22	30

SD- Standard Deviation, CV- Coefficient Variation.

Effect on Whiteness and Yellowness

Enzymatic removal of wool impurities by the process of bioscouring resulted in whiteness improvement. Though, compared to alkaline scouring yellowness index of the bioscoured wool was less, whiteness index of both alkaline (B+S+A+E) scoured and protease (B+E) scoured wool was almost similar (Table. 3). Protease being substrate specific, it only attacks peptide bonds and does not remove colouring matter in wool whereas caustic soda removes colouring matter being non-specific in action.

Table 3 Wool fiber properties at different stages of textile wet processing

Serial no.	Fibre sample	WI	YI	BI
1	Raw wool	8.71	10.31	38.51
2	Alkaline scouring	21.29	20.46	49.57
3	Protease Scouring (B+E)	20.76	11.37	48.27
4	B+S+ E	12.19	16.96	50.96
5	B+A+E	16.29	15.56	52.43

6	B+S+A+E	17.21	14.31	48.01

B= Buffer, S= Soap, A= Alkali (sodium carbonate), E= Enzyme, WI- whiteness index, YI- yellowness index, BI- brightness index.

FTIR Spectroscopic Analysis

The FTIR spectra of the raw wool, alkaline-scoured, protease-scoured fibers were shown in (Figure. 6 to 10) indicating characteristic absorption bands assigned mainly to the peptide bonds (CONH) which represent the fundamental structural unit of the polypeptide chain **(Zoccola *et al*., 2009)**. The results confirm that no new chemical bonds are produced in wool fibre. The two sharp peaks in the range of 2935–2915/2865–2845 cm^{-1} for raw wool fibre samples corresponds to Methylene C-H asymmetric/symmetric stretch and similarly at 1485–1445 cm^{-1} for Methylene C-H bend **(Meyers, 2000)**. Intensity of these peaks in raw wool fibre is high, while after protease treatment the intensity of all these peaks (Methylene C-H stretch and bend) reduces, indicating the removal of 18-MEA acid covalently bonded to epicuticle. The peak at 1737 cm^{-1} for raw wool fibre corresponds to covalent bond of 18-MEA acid to epicuticle through the (C=O) sulfoester. After B+E solution (protease) treatment, this bond breaks and it can be seen from Fig.7 that, peak at 1737 cm^{-1} disappears. The five spectra reveal that the bands near 1600cm^{-1} assigned to amide I and II vibrations are shifted. They reveal a combination of amide C=O and N-H modes. The frequency is sensitive to protein conformation, i.e., alpha helix, random, beta-sheet, etc. The intensity is proportional to the concentration of amide linkage, i.e.,-C (=O)-N (-H)-. Yet in this case, it is suspected that the differences are ascribed to the differences in the water content of fibers. There is an H-O-H bending mode at 1635 cm^{-1}. This is supposed to push up the intensity of the amide I peak after B+E solution (protease) treatment **(Mori and Inagaki, 2006)**. After protease treatment the additional peak appears at 1076 cm^{-1} which corresponds to the S-S oxidation in the surface of wool after treatment **(Meyers, 2000; Hocker, 2002)**.

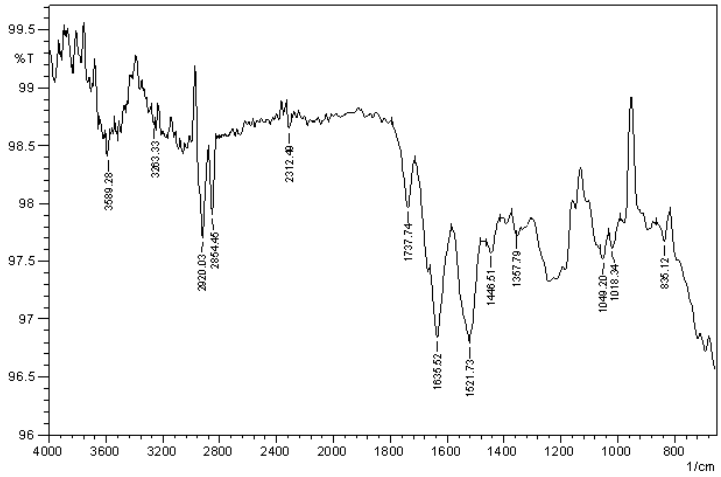

Figure 6 FTIR spectra of raw wool fibre
Analysis of functional group of raw wool fibre at wavelength ranging from 750 cm^{-1} to 3600 cm^{-1}.

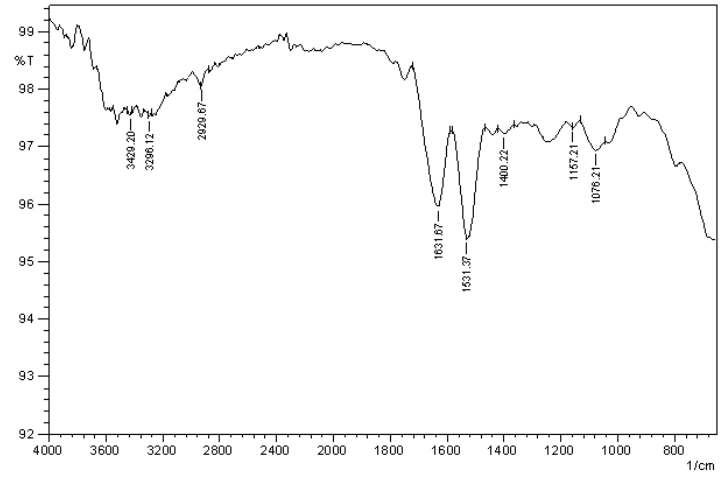

Figure 7 FTIR spectra of protease scoured (B+E) wool fibre.
Analysis of functional group of protease scoured wool fibre at wavelength ranging from 750 cm^{-1} to 3600 cm^{-1}.

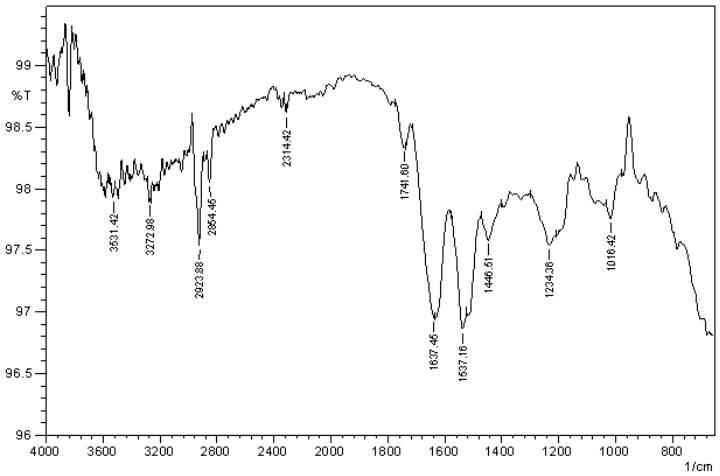

Figure 8 FTIR spectra of buffer, soap, soda ash and enzyme (B+S+S+E) scoured wool fibre.
Analysis of functional group of B+S+S+E scoured wool fibre at wavelength ranging from 750 cm^{-1} to 3600 cm^{-1}.

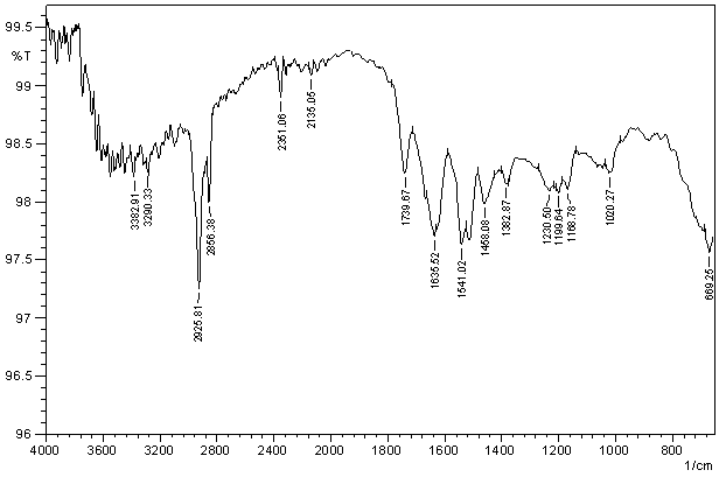

Figure 9 FTIR spectra of buffer, soda ash and enzyme (B+S+A+E) scoured wool fibre.
Analysis of functional group of B+S+A+E scoured wool fibre at wavelength ranging from 750 cm^{-1} to 3600 cm^{-1}.

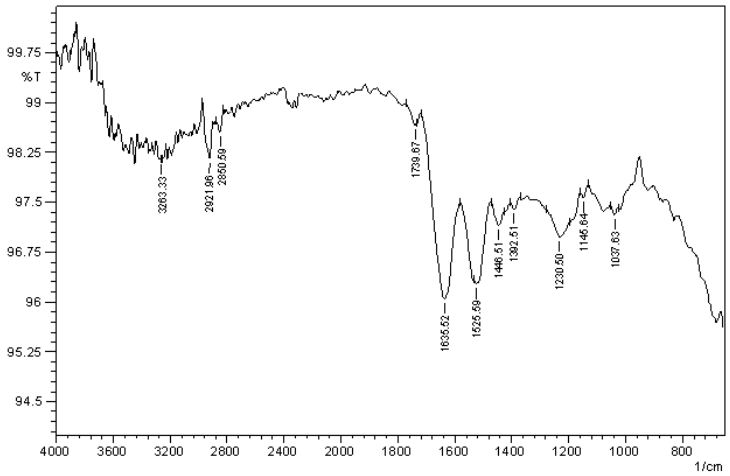

Figure 10 FTIR spectra of buffer, soap and enzyme (B+S+E) scoured wool fibre.
Analysis of functional group of B+S+E scoured wool fibre at wavelength ranging from 750 cm^{-1} to 3600 cm^{-1}

SEM ANALYSIS

The clarity of wool surface was observed with scanning electronic microscope (SEM), as shown in (Figures 11 to 15). It could be seen that, the wool surface scoured using protease enzyme (B+E solution) was very clean and smooth as compared with those scoured using conventional process, B+S+E solution, B+A+E solution and B+S+A+E solution. There was partial removal of cuticles

from wool fibre due to substrate specificity of protease enzyme. Prominent scales were observed on wool fibre surface when treated with B+E, as it got damaged with protease enzyme treatment. There was though a rise in surface performance and capillarity effect of wool fiber under this bio-scouring.

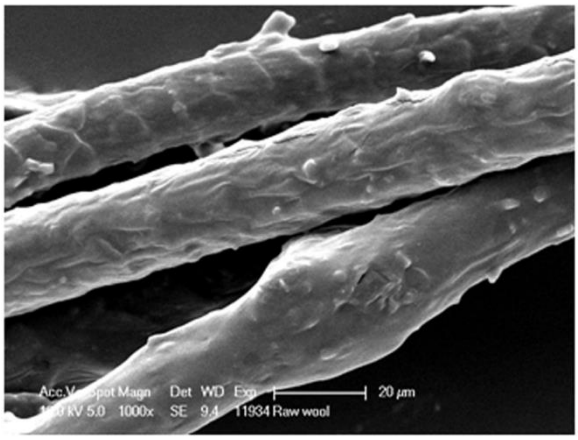

Figure 11 SEM images of raw wool fibre (1000x). Surface analysis of raw wool fibre.

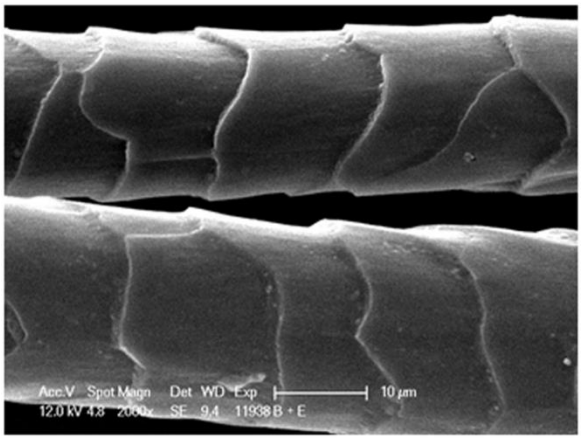

Figure 12 SEM images of protease (B+E) scoured wool fibre (2000x). Surface analysis of protease (B+E)scoured wool fibre.

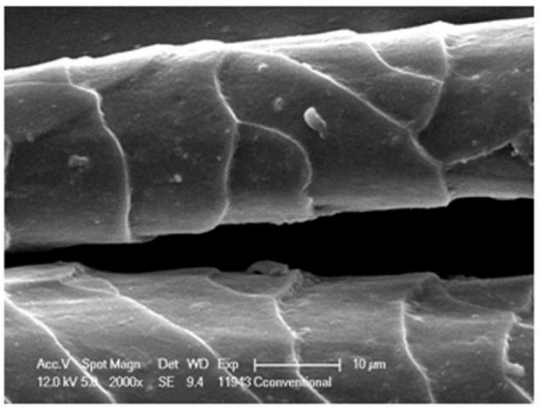

Figure 13 SEM images of alkaline(B+A+S+E) scoured wool fibre (2000x). Surface analysis of alkaline (B+A+S+E)scoured wool fibre.

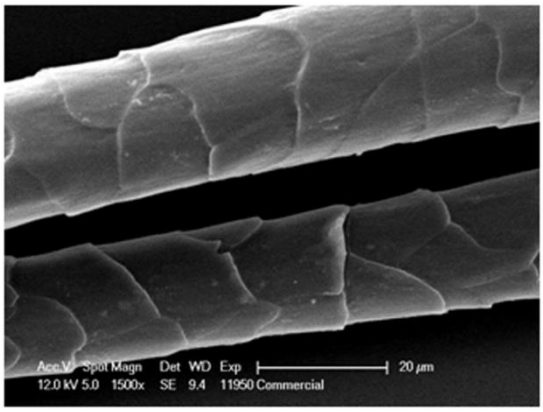

Figure 14 SEM images of commercial scoured wool fibre (1500x). Surface analysis of commercial scoured wool fibre.

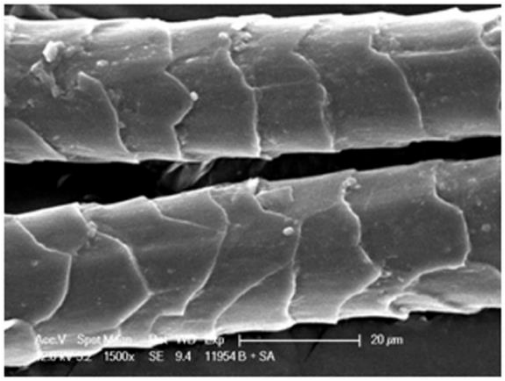

Figure 15 SEM images of B+A+E scoured wool fibre (1500x). Surface analysis of B+A+E scoured wool fibre.

CONCLUSION

The bioscouring studies indicated that protease from *B. subtilis* is capable of removing waxes from the raw wool fibre and impart hydrophilicity to the fibre. In addition, the experimantal studies of bioscouring parameters clearly states that under alkaline conditions at 60ºC for 120 min with a protease dosage of 10% on the weight of fibre, better than required hydrophilicity is achieved. The sharp peaks obtained from the FTIR data and smooth surface of wool revealed by the SEM images show the efficiency of protease in the removal of waxes from wool fibre indicating no damage to the wool fibre. Thus, the protease is a potential candidate to be used in bioscouring of wool. The approach described in the present work seems to be convincingly reproducible and environment friendly which can be easily adapted by the textile industry.The further work can be undertaken to check dyability of such bioscoured wool fibers vis-a vis conventionally scoured wool and also combine bioscoring and dyeing to acheive shortening of overall processing cycle for dyed wool.

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INHIBITION OF AN EXTRACELLULAR POLYGALACTURONASE FROM *GEOTRICHUM CANDIDUM* BY A PROTEINACEOUS INHIBITOR ISOLATED FROM LEMON FRUITS

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ABSTRACT

An extracellular polygalacturonase [EC 3.2.1.15], named PG-543, was purified from the culture of *Geotrichum candidum* IEV 543, a phytopathogenic fungi isolated from infected lemon. The molecular mass of the enzyme was estimated to be 26 kDa by SDS-PAGE. The pH optimum determined was 5.5; the enzyme showed high stability in the pH range between 4.0 and 6.0. The temperature optimum was 37 ºC. Furthermore, a proteinaceous inhibitor of the polygalacturonase (PG) named PGIP was isolated from lemon albedo. This protein was able to maintain the inhibitory activity in a wide range of pH and temperature. The maximal interaction between PG-PGIP was reached after 20 min of contact. PGIP did not show toxic effect on *Artemia salina* in the concentration range that was active on PG enzyme. Also, the proteinaceous inhibitor did not show mutagenic nor phytotoxic effects.
Our results suggest that, the PGIP isolated from lemon could be used as a natural product to decrease the propagation of pathogenic fungi responsible for postharvest diseases in citrus by the inhibition of hydrolytic enzymes secreted by pathogens.

Keywords: polygalacturonase (PG); *Geotrichum candidum*; PG-inhibitor protein; lemon fruits; PG-PGIP interaction

INTRODUCTION

Polygalacturonases (PGs) catalyze the hydrolysis of 1,4-α-D-galactoside-uronic linkages of plant cell wall pectin. PGs are widely distributed among fungi, bacteria and many types of yeasts and also found in higher plants and some plant parasitic nematodes **(Kazuo *et al.*, 2002.; Contreras Esquivel and Voget, 2004; Niture and Pant, 2004; Jacob *et al.*, 2008)**. The PGs from microbial sources have been characterized and classified into the glycoside hydrolase family 28 (http://afmb.cnrs-mrs.fr/CAZY/index.html). They are important agents of fungal pathogenicity necessary to colonize the plant tissue during their invasion. These enzymes decompose plant cell wall components causing a localized breach which constitutes the gate of infection **(Lazniewska *et al.*, 2012)**. PGs enable a pathogen to invade plant tissues, and also, their activity may trigger plant defense responses. Plants possess numerous enzymes inhibitors, such as polygalacturonase inhibitor proteins (PGIPs) which may protect them from destructive effects of fungal hydrolases **(Lagaert *et al.*, 2009; Protsenko *et al.*, 2010; Schacht *et al.*, 2011)**. Besides PGIPs, there are inhibitors of pectin lyases, pectin methylesterases, pectate lyases, endoxylanases, xyloglucan-specific endoglucanases, whose activities, functions and distribution among plant species have been described **(Favaron *et al.*, 1994; Isla *et al.*, 2002; Faize *et al.*, 2003; Lagaert *et al.*, 2009)**.
Geotrichum candidum Link: Fr. is a yeast-like fungus that causes diseases in humans, animals and plants **(Carmichael, 1957; Butler, 1960; Butler *et al.*, 1965; Suprapta *et al.*, 1996)**. This fungus has been reported from many citrus growing areas of the world **(Brown and Eckert, 1988; Hershenhorn *et al.*, 1992)**. It causes sour rot in citrus fruits, which is an important cause of postharvest disease and economic losses **(Nakamura *et al.*, 2008)**. The fungus penetrates the fruit only through injuries, particularly deep injuries that extend into the albedo. These injuries may be caused by insects or mechanical means, such as thorn or stem punctures, or by plugging at harvest. This disease is one of the main causes of postharvest losses of citrus in Tucumán province (Argentina), which is a leading citrus producing region in the world; particularly of lemon and lemon industrial products such as concentrated lemon juice, lemon essential oil and dried lemon peel. The destinations for fresh fruit are the European Union, Russia, Ukraine, Japan, Canada, Hong Kong, Southeast Asia, and Asia Minor and for industrialized products, United States, Canada, European Union, Japan, Australia and Israel (Asociación Tucumana de Citrus, 2013). Synthetic fungicides have a major role in order to reduce postharvest losses due to phytopathogenic fungi. However, problems resulting from their extensive use, as well as their associated health and environmental risks, have promoted the search for new and safer alternatives. Natural products capable of inhibiting fungi hydrolytic enzymes could be important tools in preventing these kinds of infections. In the present work we report the purification and biochemical characterization of a PG from a citrus pathogen *G. candidum* strain. Also, the effect of a glycosidase inhibitory protein isolated from lemon fruits on the purified PG was evaluated.

MATERIALS AND METHODS

Reagents

All reagents used were analytical grade and purchase from Merck & Co. or Sigma Aldrich Argentina.

Production, purification and characterization of polygalacturonase enzyme from *Geotrichum candidum*

Microorganism

The *G. candidum* strain (IEV 543) was isolated from infected citrus fruits in order to support their pathogenicity and then identified by the Laboratory of Mycology from Facultad de Bioquímica, Química y Farmacia (UNT). This isolate was routinely cultured on potato dextrose agar (PDA, Merck) slants for preservation.

Enzyme production

The enzyme was prepared according to **Torres *et al.* (2011)** with some modifications. *G. candidum* was grown on a pectin containing medium (peptone, 10 g/L and pectin, 30 g/L), without shaking, at 28 °C for 10 days. Cell-free supernatants were obtained by centrifuging the cultures at 21,000×*g* for 15 min (4 °C). Supernatants (protein: 3.15 g/mL) were concentrated by precipitation with 100% solid $(NH_4)_2SO_4$, dialyzed against 20 mM sodium acetate buffer pH 5.5 and then used as a source of PG activity.

Enzyme purification

The crude extracts were precipitated with 100% solid $(NH_4)_2SO_4$ and concentrated six fold by ultrafiltration using an Amicon system with a membrane with a cutoff point of 10 kDa. The concentrated enzyme was applied to a DEAE-Sepharose CL 6B (10 x 1.5 cm) column equilibrated with sodium acetate buffer (0.2 mM, pH 5.5) and eluted at a flow rate of 0.5 mL/min with a linear gradient of NaCl (0–1 M). The fractions containing polygalacturonase activity were pooled and applied to a Sephadex G-150 column (20 x 2 cm) equilibrated with 20 mM sodium acetate buffer, pH 5.5 and eluted with the same buffer. The eluted fractions were monitored at 280 nm for protein and assayed for enzyme activity. The fractions showing maximum polygalacturonase activity were pooled for further studies.

Enzyme assay

The PG activity was determined by measuring the release of reducing sugars, according to the Nelson–Somogyi method (Nelson, 1944; Somogyi, 1945) using sodium polygalacturonate as substrate. The reaction mixture for standard assay contained 20 µL of enzyme preparation in 0.2 M sodium acetate buffer pH 5.5 and 0.2% (w/v) sodium polygalacturonate in a final volume of 100 µL. Enzyme reactions were started by the addition of the substrate to the reaction mixture and incubated at 37 °C at different time (10 to 180 min). The reaction was stopped by boiling the reaction mixture. One enzyme unit (EU) activity was defined as the amount of enzyme which produced 1 µmole of reducing sugars per min at pH 5.5 and 37 °C.

Determination of protein concentration

Protein quantification was carried out by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Analytical electrophoresis

SDS-PAGE (15%) was performed in order to check the homogeneity and the molecular mass of the enzyme (Laemmli, 1970). Protein bands were visualized by $AgNO_3$ staining technique.

Molecular mass estimation

The molecular weight of purified PG was estimated by SDS-PAGE (Laemmli, 1970). SDS-denatured molecular weight markers were used for the construction of the calibration curve.

Substrate specificity and mode of action of the PG

Sodium polygalacturonate and citric pectin (0.2% w/v) were used as substrate in order to evaluate the substrate specificity of the enzyme. Solutions containing reaction products were filtered through Amberlite MB-3 and concentrated by evaporation for further evaluation by TLC. The samples were spotted onto a silica gel TLC plates (Kieselgel 60 0.2mm, Merck). The plates were developed in ascending direction with butanol:pyridine:water (6:4:3 v/v) as mobile phase and revealed with diphenylamine/aniline/phosphoric acid reactive (Chaplin and Kennedy, 1986). Products containing uronic acid residues appeared as blue spots. The reaction products were also evaluated at 235 nm in order to identified lyase activity. Endo-polygalacturonase activity of PG-543 was confirmed by measuring the enzyme activity using the Ruthenium red method according to **Torres *et al.* (2011)**.

Effect of pH and temperature on enzyme activity and stability

The influence of pH on polygalacturonase activity was measured by performing the activity assay at pH values ranging from 3.0 to 10.0 at a constant temperature of 37 °C. The optimum pH of the enzyme was investigated in 40 mM citrate-phosphate (pH range 3–4), sodium acetate (pH 5.5), sodium phosphate (pH range 7–8) and glycine-NaOH (pH 10) buffers. Optimum temperature for enzyme activity was determined by varying the assay temperature from 25 to 60 °C at the optimum pH. The pH stability of the enzyme was determined by pre-incubating the enzyme in the different aforementioned buffers at the optimum temperature and measuring the residual activity after 45 min. Temperature stability was studied by incubating the enzyme at different temperatures (25–60 °C) at the optimum pH for 45 min and measuring the residual activity.

Enzyme kinetics

Kinetic constants, Km and Vmax, of the enzyme were calculated by fitting activity data with different substrate concentrations (2–30 mg/mL) to a linear regression on Lineweaver-Burk double-reciprocal plot. The enzyme concentration was kept constant.

Isolation and characterization of inhibitory protein of PG from lemon albedo

Extraction and partial purification of inhibitory protein of PG from G. candidum

Lemon fruits were washed with distilled water and peeled. Flavedo and albedo were excised and cut into small segments (approximately 1 - 5 mm^2). A sample (100 g) of albedo was homogenized with the aid of a high-speed blender, in 200 mL of sodium acetate buffer (20 mM; pH 5.5) containing NaCl (1 M) and β-mercaptoethanol (1 mM). This suspension was stirred overnight at 4 °C. Then, in order to separate the solid components, the slurry was filtered and centrifuged at 10,000x*g* for 15 min. The supernatant was carried out to 100% saturation with solid ammonium sulphate and centrifuged at 10,000x*g* for 15 min. The insoluble fraction was recovered, dialyzed against the same buffer without NaCl (3 h) and re-suspended in the same buffer. After that, the protein extract was treated with PVPP (10%) to obtain a polyphenolic-free proteinic extract. The protein fraction was applied to a DEAE-Sepharose CL 6B (10 x 1.5 cm) column equilibrated with sodium acetate buffer (200 mM, pH 5.5) and eluted at a flow rate of 0.5 mL/min with a linear gradient of NaCl (0–1 M) in the same buffer. The eluted fractions were monitored at 280 nm for protein determination and assayed for PG inhibitory activity. The fractions showing inhibitory activity were pooled for further studies. SDS-PAGE was performed as previously stated in order to check the homogeneity of the inhibitory protein.

Effect of inhibitory protein on PG

Effect of the concentration of inhibitory protein on PG activity

The PG activity was measured in a mixture containing different inhibitor concentration (0.1-1.2 µg/mL), enzyme (1.15 EU) and sodium polygalacturonate (2-30 mg/ml) as substrate in 0.2 mM sodium acetate buffer pH 5.5. The reaction mixture was maintained at 37 °C during 30 min. The PG activity was determined by measuring the release of reducing sugars, according to the Nelson–Somogyi method **(Nelson, 1944; Somogyi, 1945)**. The inhibition constant, Ki, was determined by Dixon plot.

Effect of contact time between enzyme and inhibitor on PG activity

The effect of contact time between PG and inhibitor was determined. The enzyme (1.15 EU) and inhibitory protein (0.9 µg/mL) were pre-incubated at pH 5.5 during different times (5 to 40 min). Then, the substrate (0.7 mg/ml) was added to the reaction mixture and the activity was measured as previously stated.

Effect of pH on inhibitory protein stability

The inhibitor was pre-incubated at room temperature in solutions with different pH (between 2 and 10) during 45 min. Then, the activity of inhibitory protein on PG was assayed at pH 5.5.

Effect of temperature on protein inhibitory stability

The inhibitor was preincubated at different temperatures (between 25 °C and 60 °C) for 45 min. Then, the activity of inhibitory protein against PG was assayed at pH 5.5.

Determination of protease and glycosidase activities in inhibitory protein fraction

Protease assay

Protease activity was assayed by using azocasein (Sigma-Aldrich) as substrate. The reaction mixture for standard assay contained 10 µL of PGIP fraction (0.9 µg/mL final concentration) in 40 mM sodium acetate buffer pH 5.5 and 1% (w/v) azocasein in a final volume of 350 µL. After incubation at 37 °C for 30 min, proteolysis was stopped by the addition of 1 mL trichloroacetic acid (TCA) 10%. Precipitation was achieved by cooling at 0 °C for 20 min and it was centrifuged at 10,000x*g* for 5 min. An equal volume of NaOH (1 N) was added to the supernatant and the absorbance was recorded at 440 nm.

Glycosidase assay

Glycosidase activity was assayed by using sucrose (Sigma-Aldrich) as substrate. The reaction mixture for standard assay contained 10 µL of PGIP fraction (0.9 µg/mL final concentration) in 40 mM sodium acetate buffer pH 5.5 and 1% (w/v) sucrose in a final volume of 110 µL. After incubation at 37 °C for 30 min, glucose produced from the hydrolysis of sucrose was measured adding 1 ml of reagent A (Enzymatic glycemia AA, Wiener Lab., Argentina) containing glucose oxidase (10 kU/l), peroxidase (1 kU/l), 4-aminofenazone (0,5 mM), PBS (100

mM, pH 7) and 4-hydroxibenzoate (12 mM). After 5 min at room temperature the absorbance was recorded at 505 nm.

Toxicity and genotoxicity assay of inhibitory protein

General toxicity of protein using Artemia salina

The *A. salina* lethality test was employed as a general toxicity test, using lethal concentration (LC_{50}) as an indicator of the short-term poisoning potential. The LC_{50} value was defined as the amount of inhibitor that causes the death of 50% of nauplii. *A. salina* encysted eggs were incubated in 50 mL of filtered sea water in a small container divided into two compartments. The shrimp eggs were added to the covered compartment and a lamp was placed above the open side of the container in order to attract hatched shrimps through perforations in the partition wall. After 24 h incubation, the mature shrimps (nauplii) were collected with a Pasteur pipette. Ten nauplii were added to well of 96-well plate containing a dilution of the inhibitory protein (between 0 and 5 µg/mL). Control treatment containing only sea water was included in the experiment under the same conditions used in the others treatments. After 24 h incubation under light, the number of dead and surviving brine shrimps in each well was counted. The accuracy of the method was evaluated using at least three replicates of each concentration of protein. The positive control was potassium dichromate.

Phytotoxicity assay

Lactuca sativa L. var. Gran Rapid seeds were selected for seed germination and elongation assays. Seeds were placed on filter paper disks (Whatman No. 1) set at the bottom of 24 well-plates (10 per well) and treated with different inhibitor concentrations (up to 5 µg of protein). Growth tests were carried out using seedlings with primary roots equal to 2 cm. The seedlings were placed on Petri dishes (10 per well). Negative controls were performed with sterilized distilled water. Petri dishes from germination and growth treatments were maintained for 120 h and 48 h, respectively, at 25 ± 1°C. Treatments were replicated four times.

Genotoxicity assay using the Ames test

Genotoxicity assays were performed following the procedure described by **Maron and Ames (1983)**. *Salmonella thyphimurium* TA98 and TA100 were cultured overnight in Oxoid Nutrient Broth for 12 h. Different dilutions of inhibitory protein were added to 2 mL of top agar and 0.1 mL of each bacterial culture, and poured onto a plate containing minimum agar. The plates were incubated at 37 °C for 48 h and the histidine revertant colonies were counted. All experiments were performed in triplicate with at least two replicates. A two-fold or greater increase in the number of revertants exposed to the test material over spontaneous reversion rate was considered a positive mutagenic result. Negative and positive controls were included in each assay. The mutagen used as positive control was 4-nitro-*o*-phenylenediamine (10 µg per plate), which is a direct-acting mutagen.

RESULTS AND DISCUSSION

Isolation and characterization of PG

PGs, the most abundant and extensively studied of the pectinolytic enzymes, typically exist in multi-gene families and may have both *endo* **(Benen *et al.*, 1999)** and *exo* activities **(Sakamoto *et al.*, 2002)**. Previously, endo-PG production by *G. candidum* was reported by **Barash *et al.* (1984)**. Nevertheless, recently **Nakamura *et al.* (2008)** reported that there are two *G. candidum* types: pathogenic and non-pathogenic isolated. In addition, they reported that PGs from *G. candidum* citrus type were responsible for its pathogenicity to citrus fruits **(Nakamura *et al.*, 2003)**. But, PGs from *G. candidum* non-citrus type had no pathogenicity (affinity to protopectin; protopectinase activity) **(Nakamura *et al.*, 2003)**. In this work, in order to distinguish between the citrus and non-citrus types of *G. candidum*, a first pathogenicity test (inoculating the fruit with the fungus) was carried out. Then, *G. candidum* isolated from infected fruit was cultured in a media with pectin in order to promote the PG production. The highest polygalacturonase activity was obtained after 10 days of incubation of the fungus. PG-543 was purified to homogeneity from the culture supernatant of *G. candidum* after three steps of purification that included ion-exchange and size exclusion chromatographic techniques (Table 1).

Table 1 Purification scheme of polygalacturonase from *G. candidum.*

	Volume (ml)	Activity EU/ml	Protein µg/ml	Specific activity (EU/mg)	Purification (fold)	Yield (%)
Crude extract	280	914.46	178.88	5.11	1.0	100.0
Amonium sulphate precipitation	12	1540.19	229.40	6.71	1.3	7.2
DEAE Sepharose CL6B	39	444.51	5.72	77.71	15.2	6.8
Sephadex G-150	75	231.30	2.36	98.01	19.2	6.8

The enzyme was recovered from the liquid culture medium by ammonium sulphate precipitation. Then, two sequential chromatographic steps in DEAE-Sepharose CL6B and Sephadex G-150 (Table 1) were performed and one peak of PG-543 activity was obtained after last step of purification (Fig. 1.A and 1.B).

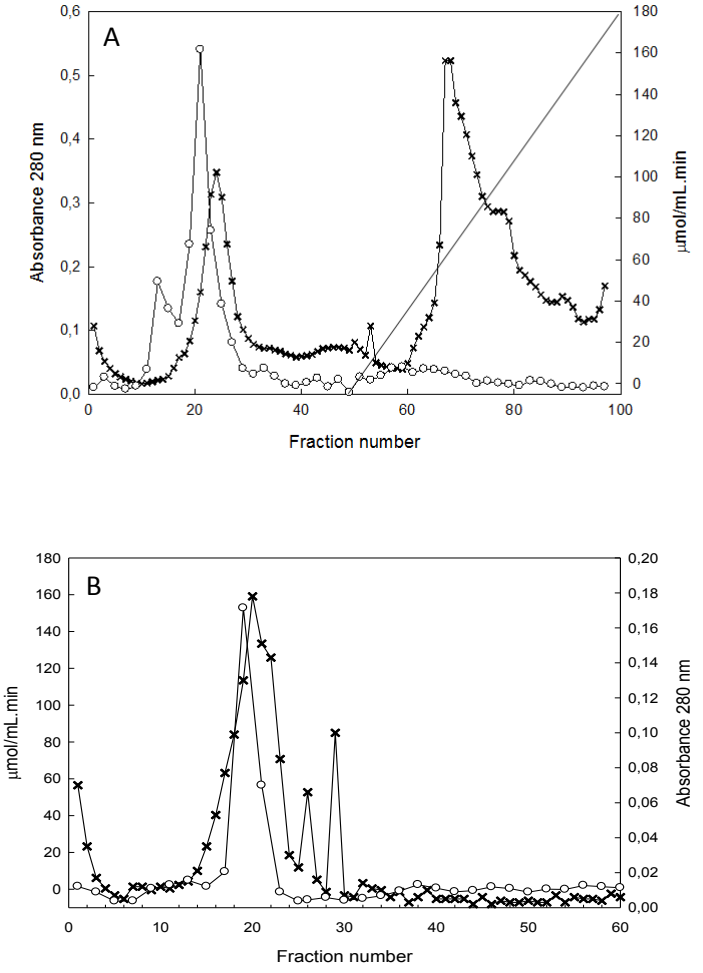


Figure 1. Elution profile of PG-543 from *G. candidum* in (A) DEAE-Sepharose CL 6B column (10 x 1.5 cm), eluted with a linear gradient of NaCl (0–1 M); (B) Sephadex G-150 column 20 cm×2 cm i.d.), eluted with 50mM sodium acetate buffer, pH 5.5. -○- PG activity (µmol/mL.min), -x- absorbance at 280 nm.

The enzyme was purified about 19-fold with an increase in specific activity up to 98 EU/mg, which represented a 6.8% yield (Table 1). SDS-PAGE analysis revealed the presence of a polypeptidic band of approximately 26 kDa (Fig. 2).

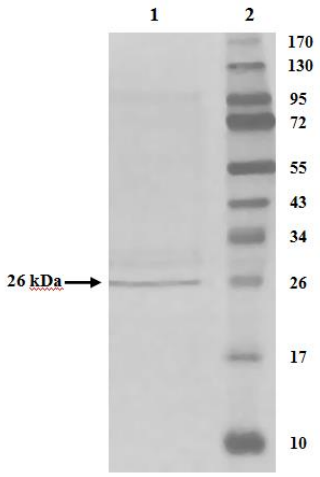


Figure 2 SDS-PAGE (15%) analysis of the purified PG-543 from *G. candidum*. Proteins were stained using the silver staining procedure. Lane 1: Fraction from Sephadex G-150 column showing purified PG-543 (1 µg of protein). Lane 2: Molecular mass standard. The molecular mass standards are reported in kDa.

Similar results were reported for the PGs produced by other strains of *G. candidum*. **Barash *et al.* (1984)** reported that the MW of the endo-PG from *G. candidum* was 38 kDa. **Nakamura *et al.* (2001)** also reported that the presumptive MW of PG S31PG1 from *G. candidum* citrus race S31 was 35.5 kDa on the basis of its amino acid sequence. Generally, PGs isolated from different microorganisms showed molecular weights around 30 kDa **(Gadre *et al.*, 2003; Niture and Pant, 2004; Mohamed *et al.*, 2006; Yuan *et al.*, 2012)**.

The purified PG showed maximum activity at pH 5.5 and at a temperature of 37 °C. The effect of pH on the stability of PG-543 from *G. candidum* was investigated by incubating the enzyme at different pH values for 45 min. The results showed that the enzyme was very stable at pH 5.5, but did not retain its activity at lower and higher pH values. These results are consistent with those reported for many microbial PGs **(Serrat *et al.*, 2002; Aminzadeh *et al.*, 2006; Mohamed *et al.*, 2006; Dinu *et al.*, 2007; Kant *et al.*, 2013)**. In general, most PGs are acidic and/or neutral enzymes (optimal pH between 3 and 6.5).

The purified PG-543 showed and optimum temperature of 37 °C and was stable at low and media temperatures (20 to 45 °C), but was severely affected at higher temperatures (Fig. 3). These results are similar to those informed for the PGs from fungi *Tetracoccosporium* sp. and *Trichoderma harzianum*, with optimal temperatures of 40 °C **(Aminzadeh *et al.*, 2006; Mohamed *et al.*, 2006)**. However, other authors reported optimum temperatures for PG activities between 50 and 55 °C **(Serrat *et al.*, 2002; Cho *et al.*, 2012; Maller *et al.*, 2013; Yadav *et al.*, 2013)**.

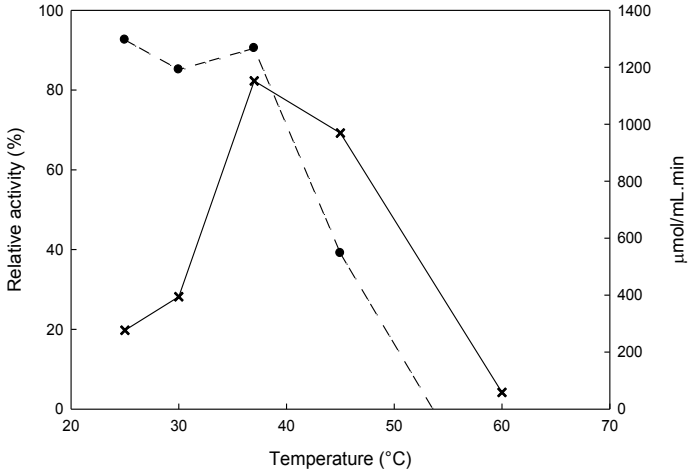


Figure 3 Temperature optimum (-x-) and temperature stability (-●-) of PG-543.

The study of substrate specificity for *G. candidum* PG-543 was made by using citric pectin and sodium polygalacturonate as substrates. The results showed that PG-543 was specific only toward sodium polygalacturonate. Analysis of the hydrolysis products by TLC and enzyme activity assay using Ruthenium red method confirmed endo-polygalacturonase activity of PG-543. The kinetic constants of the purified PG were calculated by fitting the data to a linear regression on double-reciprocal plot. The kinetic was Michaelian with linear Lineweaver–Burk plot (data not shown). K_m value for sodium polygalacturonate was 2.94 mg/mL and the corresponding V_{max} was 227.3 µmol/mL min. The estimated K_{cat} was 41.7 s^{-1} (Table 2).

Table 2 Kinetic parameters of *G. candidum* PG-543[a] in the absence and presence of PGIP from lemon.

Sample	Kinetic Parameters				
	K_m (mg mL^{-1})	V_{max} (µmol mL^{-1} min^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m (mg mL^{-1} s^{-1})	K_i (µg mL^{-1})
G. candidum PG-543	2.94	227.3	41.7	14.18	N.D.[c]
G. candidum PG-543 + PGIP	2.94	11.7[b]	N.D.	N.D.	0.125

[a] The PG activity was measured in a mixture containing PG-543 enzyme (1.15 EU) and sodium polygalacturonate (2-30 mg/mL) as substrate in 0.2 M sodium acetate (pH 5.5; 37°C), and containing or not different PGIP concentration (0.2-1.2 µg/mL).
[b] 1.2 µg/mL of PGIP
[c] N.D., not determined.

In previous studies, the K_m values for fungal endo-PGs were reported between 0.08 and 14.0 mg/mL using sodium polygalacturonate as substrate **(Gummadi and Panda, 2003; Mohamed *et al.*, 2006; Yang *et al.*, 2011; Kant *et al.*, 2013)**. The K_m value of PG-543 using sodium polygalacturonate was near to K_m values ranged from 2.5 to 4.1 mg/mL described for fungal endo-PGs from *Aspergillus niger* **(Mohamed *et al.*, 2006; Arotupin *et al.*, 2012)** and *Fusarium oxysporum* **(Al-Najada *et al.*, 2012)**.

Isolation of Polygalacturonase-inhibiting protein (PGIP) from lemon albedo and characterization of the PGIP-PG interaction

As a first approach to study the disease resistance mechanism in citrus, we focused on the study of polygalacturonase-inhibiting proteins (PGIPs). These proteins inhibit the activity of fungal PG, which are considered to be an important factor for pathogenesis **(De Lorenzo *et al.*, 2001; Reignault *et al.*, 2008)**. PGIPs are cell wall proteins of vegetative as well as fruit tissues **(Favaron *et al.*, 1994; Isla *et al.*, 2002; Schacht *et al.*, 2011; Ordoñez *et al.*, 2012)**. In the present work we showed the isolation of PGIP from lemon albedo. PGIP was partially purified in order to remove compounds that may interfere with the inhibition of the PG by PGIP. The PGIP was extracted from ripe lemon albedo and was partially purified by DEAE-Sepharose chromatography. The protein fraction displaying PGIP activity was submitted to SDS–PAGE analysis. Protein bands with estimated molecular masses from 20 to 35 kDa and 70 kDa were observed (Fig. 4).

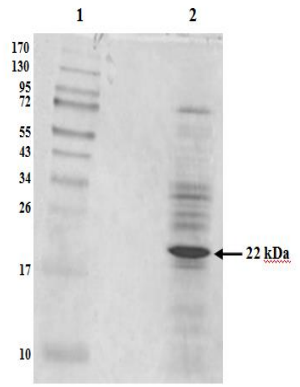
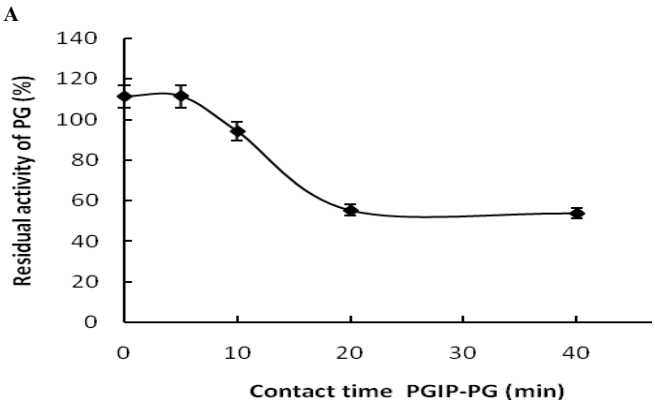
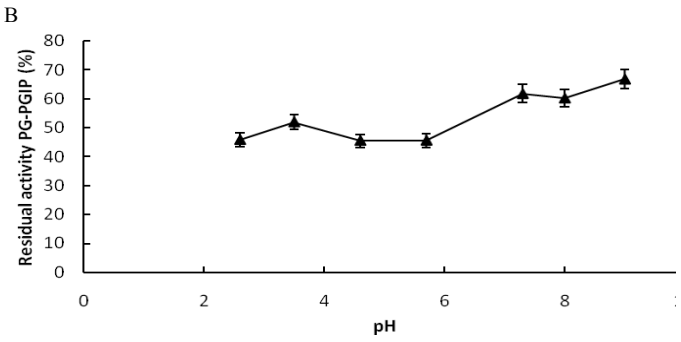


Figure 4 SDS-PAGE (15 %) analysis of partially purified PGIP extract from lemon albedo. Proteins were stained using the silver staining procedure. Lane 1: Molecular mass standard. The molecular mass standards are reported in kDa. Lane 2: Fraction from DEAE-Sepharose CL 6B column containing PGIP.

The main polypeptidic band showed a molecular mass around 22 kDa. Molecular weights of PGIPs isolated from different sources are from 30 to 70 kDa **(James and Dubery, 2001; Katoh *et al.*, 2007; Fan *et al.*, 2010)**. A previous work described a PGIP of about 70 kDa isolated from citrus leaves that was capable of inhibiting polygalacturonases from *Aspergillus niger* (**Katoh *et al.*, 2007)**. The PGIP from lemon albedo was able to inhibit completely the activity of the *G. candidum* PG-543. The contact time between PGIP (0.9 µg/ml) and PG-543 enzyme (1.15 EU) necessary to produce 50% inhibition was between 20 and 40 min (Fig. 6.A). Since PGIP was partially purified, the presence of enzyme activities that could hydrolyze PG-543 was studied in fraction displaying PGIP activity. Both protease and glycosidase activities were not detected under the same conditions able to produce 50% inhibition of PG (data not shown). The absence of hydrolytic activities in PGIP fraction discards that the loss of PG activity was due to enzymatic degradation of the PG. Previous studies reported that PGIPs from citrus leaves and pearl millet were able to inhibit 60% and 38%, respectively, the activity of *Aspergillus niger* PGs **(Katoh *et al.*, 2007; Prabhu *et al.*, 2012)**. The PGIP changed V_{max} of the enzyme (1.2 µg/mL of PGIP caused a reduction of about 90 % in the V_{max} of PG-543) but not affected K_m values of PG-543, suggesting that this protein acts as a noncompetitive inhibitor of the enzyme (K_i=0.125 µg/mL) (Table 2). Such like type of inhibition was described for PGIPs from beans and raspberry **(Lafitte *et al.*, 1984; Johnston *et al.*, 1993)**. Though, different mechanisms of inhibition were reported for PGIPs, such as an apple PGIP that showed mixed-type inhibition and the competitive inhibition of pear PGIP **(Yao *et al.*, 1995; Isla *et al.*, 2002)**.

The stability of PGIP was determined by incubating the inhibitor at different pH during 10 min and then, the inhibitor was put in contact with the PG-543 enzyme (Fig. 5.B). In these conditions, PGIP was stable in the pH range between 2 and 6, while at pH values greater than 6 the inhibitory capacity decreased. The PGIP was stable in the temperature range between 20 and 60 °C, demonstrating the thermal stability of this protein (Fig. 5.C).





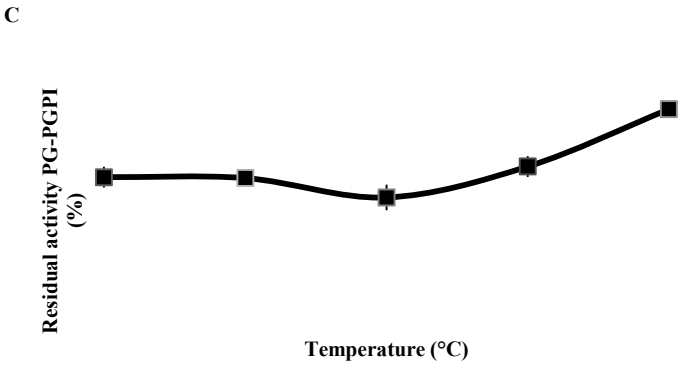


Figure 5 (A) Effect of interaction time between PGIP and PG-543 on the enzyme activity inhibition. (B) pH **s**tability of PGIP. (C) Thermal stability of PGIP.

Toxicity, phytotoxicity and genotoxicity studies

PGIP did not show toxic effect on *Artemia salina* at the concentration range in which it was active on PG enzyme. The PGIP was also assayed on germination of *Lactuca sativa* seeds and elongation of primary root. The germination was not affected by PGIP (up to 5 µg/ml). The growth of primary root in presence of PGIP was higher than the growth in its absence. Furthermore, mutagenicity was evaluated by the Ames assay. Table 3 shows the number of revertants/plate after the treatments with the isolated protein in two different strains of *S. typhimurium*. None of the doses (up to 5 µg/mL) were mutagenic in strains TA98 or TA100 under the conditions used in this assay. This result indicates the inexistence of mutagens that cause base pair substitution (detected in TA100) and frameshift (detected in TA98) mutations. The absence of mutagenicity for the protein preparations studied in the tested *Salmonella* strains indicates that DNA does not seem to be a relevant target for PGIP.

Table 3 Revertant/plate in the strains TA98 and TA100 of *Salmonella typhimurium* after treatment with various doses of PGPI.

Sample	µg /Plate	TA 98	TA 100
		Number of revertant/plate[a]	
PGIP[o]	1.25	25 ± 2	150 ± 10
	2.50	26 ± 3	158 ± 13
	5.00	33 ± 2	168 ± 32
Positive control[b]		1078 ± 89	963 ± 62
Negative control[c]		23 ± 1	133 ± 12

[a]Mean number of revertants [Mean of four plates ± S.D.]
[b]Mean number of revertants induced by reference mutagens 4-nitro-o-phenylenediamine (10 µg/plate) positive control
[c]The number of spontaneus revertants was determined in a assays without sample.

CONCLUSIONS

Plant cell wall-degrading enzymes are present in both pathogenic and nonpathogenic microorganisms and the role of each enzyme in infection depends on the combination of pathogen and host. In certain situations, some enzymes could be important for infection and others not. However, scientific evidence demonstrated that one type of cell wall-degrading enzyme, the PGs, play an essential role in the pathogenicity of pathogens that infect pectin-rich plants **(Isshiki *et al.*, 2000; Nakamura *et al.*, 2003)**. *G. candidum* IEV 543 was able to produce a PG that catalyzes a hydrolytic cleavage of galacturonic acid polymers with low C-6 methylation grade (pectate). PG-543 could be a very important virulence factor in *G. candidum* that enables infection of fruit tissue. We have

shown that the proteinaceous inhibitor isolated from "lemon albedo" inhibited this enzyme. PGIP is suggested to be involved in the defense mechanism of lemon to *G. candidum*, performing as a hydrolase inhibitory protein. This PGIP could be used as a natural product to decrease the propagation of this pathogenic fungus, preventing the development of postharvest diseases.

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CURING EFFECTS ON THE GROWTH OF *LISTERIA MONOCYTOGENES* AND *ESCHERICHIA COLI* O157:H7 IN CAMEL MEAT USING MOST PROBABLE NUMBER-POLYMERASE CHAIN REACTION METHOD

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ABSTRACT

MPN-PCR method was used to determine *Escherichia coli O157:H7* and *Listeria monocytogenes* in cured camel meat samples during nine days of storage. The MPN-PCR is based on the combination between the conventional Most Probable Number and the Polymerase Chain Reaction technique targeting the *Stx1*and *Stx2* gene for *Ecoli O157:H7* and *hlyA* gene for *L. monocytogenes*.Growth of both bacteria in the cured and control experimental groups was initially subjected to a spectrophotometric MPN assay using a microplate reader followed by confirming by two PCR assays. It was shown that in the inoculated samples, the population of both microorganisms was significantly increased at all the test times. However, a significant reduction in the populations of both microorganisms was shown in the cured meat compared to the inoculated -but not- cured group. Our results suggested that curing can improve the microbial quality of camel meat.

Keywords: *Listeria monocytogenes, Escherichia coli O157:H7*, *Curing, Camel meat*

INTRODUCTION

One of the major concerns in the food industry is the preservation of meat and meat products **(Drosinos *et al.*, 2006)**. Highly perishable foods such as meat provide excellent conditions for the growth of hazardous microorganisms. *Listeria monocytogenes and Escherichia coli O157H7* are the most important food-borne pathogens of humans **(Adzitey and Huda, 2010; Park *et al.*, 1999)**. *L. monocytogenes* is a Gram-positive and ubiquitous bacterium responsible for foodborne disease (Listeriosis). The bacterium can contaminate several categories of foods. Multiplying at low temperatures makes *L. monocytogenes*a difficult pathogen to control in refrigerated foods that are consumed without further lethality treatments, such as reheating. **(Delia and Silvia, 2009)**. *E. coli* O157:H7 is a foodborne pathogen with a low infectious dose that has been associated with meat and meat products **(Sivapalasingam *et al.*, 2004).**The infection can lead to hemolytic uremic syndrome (HUS), characterized by hemolytic anemia, thrombocytopenia, and renal injury **(Banatvala *et al.*, 2001).** The bacterium has the ability to survive at refrigeration temperatures **(Perez Rodriguez *et al.*, 2011).** Several methods such as cooking, fermenting, drying and curing have been used to preserve meat. Curing is one of the oldest methods for preserving and protecting meat from spoilage. Meat curing is the application of salt, color fixing ingredients, and seasoning to inform unique properties to the product. Salt and nitrite are two main ingredients used to cure meat **(Gheisari and Danesh, 2012).**

Camel is one of the most popular domestic animals in the dry and semi dry regions **(Knoess, 1977)**. Due to lower fat and cholesterol content and relatively higher polyunsaturated fatty acids, camel meat is healthier than beef **(Kadim*et al.*, 2008; Dawood and Alkanhal, 1995)**. Camel meat has a good capability for curing and additionally, mixed methods of curing resulted in more tender and higher quality than other methods **(Gheisari and Danesh, 2012)**. There are no reports on the microbiological safety of the cured camel meat. Therefore, we determined the survival of *E. coli O157:H7* and *L. monocytogenes* during the process of curing. Several methods have been employed to detect and enumerate the spoilage producing bacteria in foods. PCR is the most sensitive of rapid tests to detect microbial pathogens in samples **(Yamamoto, 2002)**. In addition, the combination of MPN and PCR techniques (MPN-PCR procedure) is a rapid detection test with advantages of less work load and less material consumption **(Chang *et al.*, 2013).**

MATERIAL AND METHODS

Bacterial cultures

A proven strain of *L. monocytogene*s (ATCC 19115) and nalidixic acid-resistant strain of *E. coli* O157:H7 (ATCC 43895) were provided by Department of Microbiology, School of Veterinary Medicine, Shiraz University. To confirm nalidixic acid-resistant *E. coli O157:H7*, the bacteria was cultred onto the McConkey agar (MERK, Germany) plate containing 400µg ml^{-1} nalidixic acid and incubated at 37°C for 24 h. Appearance of a colony was considered as bacterial resistance.

Growth curve of bacteria

L. monocytogenes and *E. coli O157:H7* were cultured in Tripticase Soy Broth (TSB) (Merck, Germany) and incubated at 37°C for 24 h. Bacterial suspension were then centrifuged at 3000g for 5 minutes. 10 ml of sterilized peptone water was added to the sediment. The process was repeated twice. Finally, 10 ml of TSB was added to the sediment, serial decimal dilutions (10^{-1}, 10^{-2}, 10^{-3}, $10^{-4}$and 10^{-5}) were prepared and the absorbance of each dilution was measured by spectrophotometer (JENWAY, U.K). 100 µL of each dilution was subsequently cultured to TSA medium and incubated at 37°C for 24 h. Number of colonies was then recorded. Bacterial growth curve was plotted based on the absorbance and number of colonies for each concentration**.**

Sampling procedure

Six male, adult Iranian dromedary (one-humped camel), were slaughtered at local slaughterhouse. In order to complete the rigor mortis, the carcasses were stored refrigerated for 24h . The *Longissimus dorsi* muscles were dissected and trimmed off external fat and connective tissue. After that, meats were chopped into pieces about 1 cm. In order to evaluate each of the bacterium, camel meat samples were divided into the following experimental groups:

Group 1: No treated samples (Negative Control).
Group 2: No curing meat containing the bacteria (*E.coli O157:H7*and/or *L. monocytogenes*) (Positive Control).
Group 3: Cured meat containing the bacteria (*E. coli O157:H7*and/or *L. monocytogenes*).
All treatments were performed in triplicate.

Meat curing

Two kilograms of meats were mixed with curing compounds [NaCl (22 g), sodium nitrite (0.18 g), soy protein isolate (15 g), starch (50 g), spices (15 g), phosphate (26 g), water and ice (400 g) and ascorbic acid (0.2 g) for 1 kg meat] before being stored at 4 °C for 9 days. Microbial count was recorded at the days 1, 3,5 and 9. All the experiments were performed in triplicate.

Inoculation of the bacteria

Each bacterium was inoculated in TSB medium and following a 24h incubation at 37°C, the number of colonies were determined according to the growth curve of bacteria. After that, 10^4cfu/g of each bacterium was added to the meat samples (Groups 2 and 3) and mixed well.

Bacterial counting using MPN-method

Different experimental groups (containing bacteria) were homogenized to prepare a 10-fold serial dilutions using peptone water up to 10-5. 96-well microplates were used for MPN test. For detection and enumeration of *E-coli* O157:H7 and *L. monocytogenes*, double-strength McConkey broth contained nalidixic acid and Buffered Listeria Enrichment Broth (BLEB) were respectively used. 100 µL of each dilution of the homogenate was transferred into culture wells containing 100µL of growth medium.
For each sample, 3 replicate MPN assays were set up for each dilution step, and the microplates were incubated in the 37°C for 24h. After incubation, the turbid wells (*L. monocytogenes*) and/or yellow discoloration (*E-coli O157:H7*) were considered as positive. The pattern of growth was then read from the MPN table to provide the most probable number and 95% confidence interval.To confirm the MPN results, each positive MPN well was used for the surface plating on the selective agar medium. Palcam agar (Merck, Germany) and CTS-Mac (AQUALAB, Germany) were respectively used for the isolation of *L.monocytogenes* and *E-coli O157:H7*. Cultured plates were incubated at 37°C for 24h. The positive MPN results were then subjected to PCR for the detection of *Stx1*and *Stx2* genes specific for *E. coli O157:H7* and *hlyA* gene for *L. monocytogenes*.

PCR

DNA extraction

On days 0, 2, 4 and 8, 200µL of turbid broth, were stored at -20°C for further use. DNA extraction was carried out using the boiled-cell method as was previously described **(Chai *et al.*, 2007)**, with some modification. Briefly, the samples were thawed and centrifuged at 10000 rpm for 10 minutes. The supernatant was discarded; the pellet was re-suspended with 50 µL of deionized water, vortexed and boiled in a water bath (100 ^oC) for 10 minutes to lyses the cells followed by cooled on ice for 2 minutes. Samples were then centrifuged at 10,000 rpm for 10 minutes. Aliquot of the supenatant which contained the template DNA was transferred to a sterile tube and stored at -20°C until further use.

MPN-PCR for Listeria monocytogenes

One pair of primers F and R with sequence of 5'-CGGAGGTTCCGCAAAAGATG-3' and 5'- CCTCCAGAGTGATCGATGTT-3' was designed to amplify *hlyA* gene at 485bp region. PCR reaction was carried out in 25 ml reaction mixture consisting 2.5 µL PCR buffer, 1.5 µL $MgCl_2$, 0.5µL dNTPs mix, 0.5µL TaqPolymerase (PROMEGA, USA), 0.5 µL of each primer, 1.5µL DNA templates and17.5µL sterile distilled water. Amplification of DNA segment was performed in thermal cycler (APPLIED BIOSYSTEMS, USA) using thethermo-cycling conditions of initial denaturation (94ºC for 2 minutes), 35 cycles of denaturation (94ºC for 45 seconds), annealing (64ºC for 45seconds), extension (72ºC for 1 minute) followed by final extension (72ºC for 5 minutes). PCR products were loaded in 1.5% agarose gel stained with ethidium bromide (1mg. ml^{-1}) and were electrophoresis at 50mA for one hour. Gel was visualized under UV light using and photograph (SYNGENE, USA).

MPN-PCR for *E. coli O157:H7*

Two pairs of primers used in MPN-PCR were *Stx1* and *Stx2* primer. Sequences of primers used are shown in table 1. PCR reaction was carried out in 21.5 ml reaction mixture consisting 2.5µL PCR buffer, 1.25µL $MgCl_2$, 1µL dNTPs mix, 0.5 µL TaqPolymerase (Promega, USA), 0.5 µL of each primer, 1.5 µL DNA templates and 13.75µL sterile distilled water. Amplification of DNA segment was performed in thermal cycler using the thermo-cycling conditions of initial denaturation (94 ºC for 2 minutes), 35 cycles of denaturation (95 ºC for 60 seconds), annealing (64 ºC for 60seconds), and extension (72ºC for 90seconds) followed by final extension (72 ºC for 7minutes). PCR products were analyzed on gels of 1.2% agarose.

Table 1 Sequence and size of primers based on the coding from the following genes used for *E. Coli* O157H7 .

Primer name	Gene	Oligonucleotide sequence (5'-3')	Amplicon size (bp)
Stx_1F	Stx_1	ATAAATCGCCATTCGTTGACTAC	480
Stx_1R	Stx_1	AGAACGCCCACTGAGATCATC	
Stx_2F	Stx_2	GGCACTGTCTGAAACTGCTCC	510
Stx_2R	Stx_2	TCGCCAGTTATCTGACATTCTG	

Statistical analysis

The data were analyzed using the Repeated Measure ANOVA test of SAS 9/1 statistical software. Duncan's post hoc test was used to assess differences between groups. Differences were considered significant at values of $P<0.05$.

RESULTS AND DISCUSSION

Moisture, protein, fat and ash percents of camel meat samples were 73.82 ± 2.63, 23.57 ± 0.64, 4.16 ± 0.72, 1.22 ± 0.35, respectively. Microbial contamination can reduce the quality of fresh meat, shorten its shelf- life and cause economic losses and health hazards. Minimizing product contamination and delaying or inhibiting growth of spoilage and pathogenic organisms in the product are major keys for improving fresh meat shelf life and increasing consumer safety. While general cleanliness and proper sanitation are very effective, other means of controlling microbial growth in the meat products may be proven useful.The contamination of camel meat with *E. coli* O157:H7 and *L. monocytogenes* was detected by using a combination of culture method and MPN-PCR technique. *E. coli* O157:H7 was revealed as clear colony occasionally with yellow-orange halo on CTS-Mac agar and *L. monocytogenes* exhibited as gray-green colony with black halo on PALCAM agar. Results of the bacterial count by MPN method are shown in tables 2 and 3. Even though, an initial contamination to *L. monocytogenes* was obvious in our samples as shown in table 3, the curing was effective enough to significantly reduce the microorganisms in all the experimental groups**.**
Both microorganisms in all the experimental groups (except for *E.coli* O157:H7) increased during the storage time but the curing was dramatically reduced the number of them. All of the turbid samples in Buffered *Listeria* Enrichment Broth were shown a DNA fragment of the expected size **(**485bp) for *Listeria* spp. Positive samples in MPN test for *E. coli* O157:H7 produced a DNA band at 480 and 510 bp regions (figure 1).

Table 2 Details of the MPN values for *E. coli* O_{157}-H_7 (cfu/g) during 9 days of storage at 4 ºC.

Group	Days of storage			
	1	3	5	9
Group 1	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
Group 2	833.5 ± 201.64^{cA}	5143 ± 1197.18^{cB}	42475 ± 9933.06^{cC}	110000 ± 12306.38^{cD}
Group 3	317.5 ± 62.99^{bA}	445 ± 17.32^{bB}	670 ± 115.38^{bC}	3300 ± 471.31^{bD}

Values are mean ± standard deviation. Group 1: control; Group 2: No curing meat containing the bacteria; Group 3: Cured meat containing the bacteria. The different small letters in the same column indicate significant differences between groups and different large letters in the same row indicates significant differences between the experimental days ($P < 0.05$).

Table 3 MPN results of *L. monocytogenes* (cfu/g) during 9 days of storage at 4 ºC.

Group	Days of storage			
	1	3	5	9
Group 1	240 ± 11.54 ^{bA}	2375 ± 50 ^{cB}	59350 ± 11981.63 ^{cC}	40500 ± 4489.80 ^{cC}
Group 2	555.75 ± 133.53 ^{cA}	1582.5± 248.01^{bB}	4100 ± 707.55 ^{bC}	19950 ± 4015.67 ^{bD}
Group 3	33 ± 4.71 ^{aA}	45.5± 13.48 ^{aA}	23± 9.76 ^{aA}	189 ± 37.95 ^{aB}

Values are mean ± standard deviation. Group 1: control; Group 2: No curing meat containing the bacteria; Group 3: Cured meat containing the bacteria. The different small letters in the same column indicate significant differences between groups and different large letters in the same row indicates significant differences between the experimental days ($P < 0.05$).

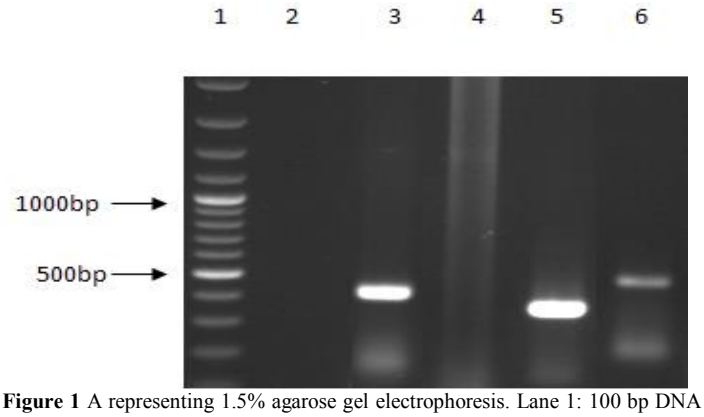

Figure 1 A representing 1.5% agarose gel electrophoresis. Lane 1: 100 bp DNA ladder; Lane 2: negative control (no template); Lane 3: 485 bp fragment corresponding to the *hly* gene of *L. monocytogenesis*; Lane 4: negative sample; Lane 5 & 6 480 bp and 510 bp fragments corresponding to the *Stx1* and *Stx2* genes of *E. coli* O157: H7

Al-Bachir and Zeinoun (2009) finde 10^6 CFU/g total (mesophilic aerobic) plate count and 10^3 CFU/g coliform count in the camel meat on day 0. This microbial load was high enough to indicate that the meat had been heavily contaminated during slaughtering and fabrication operations or alternatively, that the meat had been stored for an unknown length of time before being purchased for the study. Except for *E. coli* O157H7 in the negative control group, the bacterial populations were increased during 9 days of storage at 4 °C. The MPN method is the most advantageous to detect lower levels of microorganisms in food samples (10-100 MPN/g) **(Martin *et al.*, 2004)**. Recently, the MPN-PCR method is widely used for detection and enumeration of food-borne pathogens in various foods **(Chai *et al*., 2007; Lee *et al*., 2009).** It was also noticeable that this method is more useful and effective for detecting microorganisms such as *L. monocytogenes* and *E-coli* O157:H7 than the plating method. Thus, application of the MPN-PCR method in the present study suggest for more accurate and reliable results. Recently, more attention is paid to the nutritional value of the camel meat **(Ulmer *et al*., 2004)**. The three most common methods use for camel meat preservation and processing are thermal processing, smoking and curing **(Kalalou*et al*., 2004; Zegeye, 1999).**

Salt has a variety of effects on both food tissues and microbial cells which are responsible for its preservation action such as interactive enzyme systems vital to the cells, stopping or slowing the growth and drawing water out the cells due to osmotic pressure. Sodium chloride (NaCl) has been traditionally used in curing processes. One of the functions of NaCl in meat products is to extract myofibrillar proteins. Extraction and solubilization of these muscle proteins contributes to meat particle binding, fat emulsification, and water-holding capacity, and thus, it reduces cook losses and improves quality and texture **(Sofos, 1986).** The role of nitrite in cured meat is four-fold: i) providing the characteristic pink-red cured-meat color to the lean tissue; ii) inhibiting the growth of a number of bacteria that cause food poisoning or spoilage; iii) contributing to the distinctive flavour of cured meats; and iv) retarding the oxidative rancidity in processed meat products, principally through a process of metal chelation **(Honikel, 2008).** Results of this study showed that curing may significantly reduce the growth of *L. monocytogenes* and *E. Coli* O157: H7 in camel meat. From the first day, number of the bacteria in the treated group was less than the controls. Low pH is an important factor contributing to the inhibition growth of foodborne pathogens. Several factors including pH associate with the survival of *E. coli* and *L.monocytogenes* during fermentation of sausage **(Cho *et al*., 2011). Gheisari and Danesh (2012)** report that curing of camel meat will reduce pH values of meat. We hypothesize that change in pH contributed to the reduction of *E. coli* and *Listeria* populations during the curing of camel meat. Additionally, in a work conducted by **Castano *et al.* (2002)** the counts of Enterobacteriaceae in the sausages manufactured industrially decrease continuously from the second day after the sausage mass is stuffed.This decrease is probably due to the addition of curing salt to the samples. **Kajak and Kołożyn-Krajewska (2006)** reported that the addition of 60 ppm sodium nitrite to meat was significantly inhibited the growing of microorganisms. While **Yu and Chou (1997)** reported that sodium nitrite at the concentrations between 0.07 and 0.15 g kg-1 have no significant effect on the reduction of *E coli* O157: H7 in sausage. Camel meat is a potential source of meat particularly in the arid tropics. As we found here, curing was considerably reduced the risk of microbial contamination.

CONCLUSION

Camel meat is a potential source of meat particularly in the arid tropics. If it was cured, it would be less susceptible to the microbial contamination. However, more research work in the areas of meat production, technology, marketing, and social awareness is needed to exploit the potential of camels as a source of meat and related products.

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LACTIC ACID BACTERIA AND YEAST DIVERSITIES IN SPONTANEOUSLY FERMENTED MILLET SOURDOUGHS

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ABSTRACT

The lactic acid bacteria (LAB) and yeast diversities in spontaneously fermented pearl millet (*Pennisetum glaucum*) and finger millet (*Eleusine coracona*) sourdoughs were investigated. Pearl and finger millet grains were processed into flour and used in spontaneous sourdough fermentation. LAB and yeast cultures were isolated, screened and identified based on morphological, biochemical and sugar utilisation pattern using API 50CHL and API 20 AUX kits. Titratable acidity, pH and Temperatures of sourdoughs developed were also monitored. Titratable acidity and Temperature increased with increasing days of fermentation and conversely the pH. and mesophilic bacterial count followed that of a normal bacterial growth curve. Dominant LAB and yeasts in pearl and finger millet spontaneously developed sourdoughs were *Lb. plantarum*, *Lb. plantarum* 1, *Pediococcus pentosaseus*, *Lb. pentosus*, and *Saccharomyces cereviasiae*. However, *Candida milleri* was found in pearl millet sourdoughs. The dominant cultures in millet sourdoughs could find applications as starter cultures if improved sourdough quality is desired.

Keywords: Pearl millet, Finger millet, Sourdough, Lactic acid bacteria, Yeast, Fermentation

INTRODUCTION

Millets are group of highly variable grasses with many small seeds. Pearl millet (*Pennisetum glaucum*) and Finger millet (*Eleusine coracona*) are among the most important ones grown in the tropics but underutilized. Sourdough is an acidic pastes laden with potentials to optimize desirable functional properties in flours. It is a technological process applied to non-gluten flours. It is the foremost cereal fermentation performed with almost any cereal **(De Vust et al., 2002; Valcheva et al., 2006; Edema, 2011).** Sourdough consists of mixture of flour and clean water. Sourness is achieved by the action of fermentative organisms such as the lactic acid bacteria genera. Lactic acid bacteria and yeasts metabolic activities accounts for the desirable changes observed in sourdoughs. Some metabolites of sourdough microflora includes among other; organic acids, carbondioxide, diacetyl and alcohols.

Spontaneous fermentation is the oldest form of fermentation, it is left to chance and it is the form of fermentation in most small scale fermentations in the developing countries **(Holpzapfel, 2002)**. Spontaneous fermentations results in inconsistent product quality and hazards. Several species of microgranisms ranging from bacteria and fungi have been reported to colonize most cereal fermentations. Lactic acid bacteria (LAB) belonging to the genus - Lactobacillus have been isolated from sourdoughs and identified **(Corsetti et al., 2004)**.

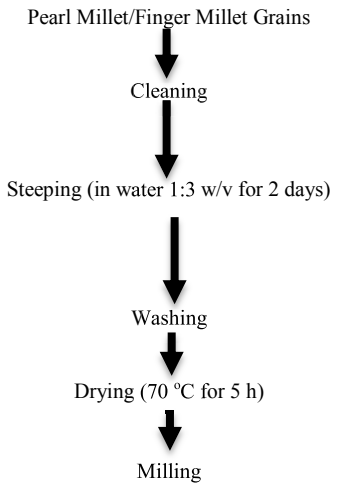
Sourdoughs are composed of diverse source of LAB and yeast species **(De Vuyst and Neysen, 2005)**. Microflora dominants in substrates are geographical region, processing and handling dependent. The LAB and yeasts developing in the dough may originate from selected natural contaminants in the flour, water, starters added or from previous fermentation vessel. LAB and yeasts are the predominant microorganisms present in sourdoughs **(Vogel et al., 1996; Steinkraus, 1996; 1997; Holzapfel et al., 1998; Lee, 1997; Oyewole, 1997)**. LAB's belong to several genera, this includes; *Lactobacillus spp., Lactococcus spp., Leuconostoc spp., Oenococcus spp., Pediococcus spp., Streptococcus spp., Tetragenococcus spp., Aerococcus spp., Carnobacterium spp., Enterococcus spp., Vagococcus spp. and Weissella spp* **(De Angelis et al., 2007)**. Great variety exist in yeast cultures found in sourdough fermentation depending on degree of dough hydration, leavening temperature, sourdough maintenance temperature and type of cereal used **(Hammes et al., 2004; Gobbetti et al., 1994)**. However, Lactobacillus and Saccharomyces genera have been reported to be the most dominant in sourdough fermentations.

Studies on the ecology of sourdough microflora may help in the understanding of the microbial dynamics and differences between groups of closely related microbial population in cereal (sourdough) fermentations. In most natural fermentation, starters used are poorly known. Therefore, isolation, characterization and identification of the microorganisms involved in cereal fermentation with prospective selection as starter cultures is important hence our investigation.

MATERIALS AND METHODS

Sample Collection

Pearl millet (*Pennisetum glaucum*) and finger millet (*Eleusine coracona*) were purchased from local market in Plateau state, Jos, Nigeria. Pearl and finger millet grains were cleaned, steeped, processed into flours and packaged in polyethylene bags for further analysis as shown in Fig. 1. The millet grains were steeped in water for two days (1:3 w/v ratios of seeds to the volume of steeping water), dried at 70 °C for 4 h in hot air oven (Laboratory oven, DHG 9101.1SA) and milled into flour using attrition mill (Atlas exclusive, Alzico Ltd mill). Flours were sieved using 60 mesh Standard US sieve and packaged.



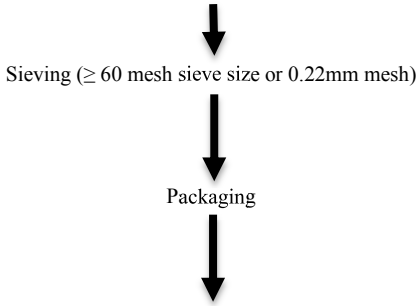


Figure 1 Production of fermented pearl/finger millet flours

Spontaneous Sourdough Development

Sourdough were developed spontaneously from pearl and finger millet flours. Equal amounts of pearl and finger millet flours (100 g) and tap water (100 ml) were mixed together using a glass stirrer in glass jars until a soupy consistency was achieved. Fermentation was allowed to proceed at ambient temperature (29±2 ^{o}C) for seven days under anaerobic condition. Equal amount of fresh aliquots (millet flour and tap water) were added to the developing batter in the jars continously using a wooden spoon. This was done each day of the sourdough fermentation in order to refresh the fermentation medium (Edema and Sanni, 2006).

Determination of pH and Temperature

Changes in pH of spontaneously developed pearl and finger millet sourdoughs were monitored with increase in days of fermentation. Twenty milliliter of spontaneously developed pearl and finger millet sourdoughs were used for the determination. The pH was determined using a pH meter (ECO Testr pH 1) after standardising using pH buffer 4.0 and 7.0 respectively. The changes in temperature of the sourdough batters was determined from the same batch used for pH determination.

Determination of titratable acidity

Titratable acid produced in the sourdoughs was determined according to method described by **Lonner et al. (1986)**. Briefly, from the same batch used for the pH and temperature determination, one (1) g of the fermenting millet sourdoughs were measured using an electronic digital balance into a conical flask and made up with distill water to 9 ml mark. Ten (10) ml each of the homogenized samples were titrated against 1 N NaOH using 2-3 drops of phenolphthalein indicator until endpoint was reached. The amount of NaOH used during the titration was expressed as total titratable acid produced. Each ml of 1N NaOH used is equivalent to 90.08 mg of lactic acid.

Microbial analysis

Enumeration and isolation of lactic acid bacteria and yeast from Sourdoughs

The microbial analysis was carried out using pour plating methods as described by **Harrigan and McCane (1976)**. From the spontaneously developed pearl and finger millet sourdoughs, one gram of each was homogenised in 9 ml sterilized saline solution (0.85% NaCl) and further diluted till a factor of nine was obtained. One (1) ml each of serially diluted homogenized samples was plated on sterilized de Mann Rogosa Sharpe (MRS) (Oxoid, PM221, USA) and Potato-dextrose (EUR Pharm, Spain) agar. Cultured MRS plates were incubated anaerobically at 30 ^{o}C for 48 h in an anaerobic jar loaded with gas pak (BBL Gas Pak, H2 & CO2; Becton-dickinson, Cockeysville, MD, USA) while that of PDA plates were incubated in a fungi incubator at 25 ^{o}C for72 h. The microbial populations of the sourdoughs were enumerated each day of fermentation. Isolation and sub-culturing was done until pure cultures were obtained. Dominant presumptive lactic acid bacteria and yeast cultures were further screened on the basis of cell morphological characteristics and biochemical (Gram reaction, catalase) characteristics using standard microbiological techniques as described by **Harrigan and McCane, (1976); Fawole and Oso (2007)**.

Gram positive and catalase negative presumptive LAB isolates were screened for nitrate, urease, indole utilisation, gas production from glucose, spore formation, growth at different temperatures (10 ^{o}C, 15 ^{o}C, 30 ^{o}C and 45 ^{o}C), NaCl salt concentrations (2%, 4% and 6.5%) and growth at different pH (4.5 and 6.0). The presumptive yeast cells were screened for growth at different NaCl salt concentration (2%, 4% and 6.5%), growth at 37 ^{o}C and utilisation of urease. Cultures were further characterised using carbohydrate fermentation pattern through the Analytical Profile Index (API Kit). The dominant presumptive lactic acid bacteria were characterised using API 50 CH and API 50 CHL medium (Biomerieux, France) while API 20 E was used for the presumptive yeast isolates. The tray was moistened with sterile distilled water to prevent drying out and inoculum (actively growing culture) was dispensed into wells after equal McFarland concentration was achieved. McFarland concentration was achieved using UV-visible spectrophotometer at 290 nm wavelenght. Anaerobiosis was achieved in the API kits by overlaying inoculated tubes on trays with sterilised paraffin oil and incubated at 30 ^{o}C for 48 h while API 20E at 36 ^{o}C for 72 h. Isolates were identified with reference to Bergey's Manual of Systematic Bacteriology **(Wood and Holzapfel, 1995)** and apiweb^{TM} identification software (Biomerieux).

RESULTS AND DISCUSSION

Sourdough paste developed spontaneously from pearl and finger millet flour had a soupy consistency as the days of fermentation increased however, more frotting was observed in sourdough developed from finger millet which might be due to the metabolic activity of yeast in the sourdough. The finger millet flour absorbed more water compared to the pearl millet flour and this might be due to higher carbohydrate and fibre content in finger millet compared to pearl millet. **Obilana (2003)** reported the carbohydrate content of whole pearl and finger millet flour as 70 g/100 g and 74 g/100 g respectively and the crude fibre content as 2.3 g/100 g and 3.6 g/100 g respectively. Higher water absorption capacity is attributed to loose structure of starch polymers **(Adebowale et al., 2008; Akinola et al., 2016)**.

Table 1 presents the morphological and biochemical characteristics of presumed lactic acid bacteria and yeast isolates from spontaneously developed pearl and finger millet sourdough. About 62.5% and 58.8% of presumptive bacteria isolates from pearl and finger millet sourdoughs were circular while 37.5 % and 41.2% were irregular in shape respectively. This findings supports the previous work of **Valík and Görner (1995) and Hutkins (2006).** The colony surfaces of presumptive bacteria isolates were generally smooth except in P3, P4, F2 and F6 that were wrinkled. However, F8 had rough surface. The colony colour of presumptive bacteria isolates ranged from white, creamy, dull white and orange in finger millet isolates. All presumptive yeast isolates were non-motile, butyrous, creamy, smooth in surface. Most isolates from pearl and finger millet sourdoughs were catalase negative. Catalase negative is indicative of the isolates ability to hydrolyse hydrogen peroxide in producing catalase enzyme. All isolates were gram positive. The morphological characteristics observed in many of the isolates corroborate the findings of previous authors on lactic acid bacteria. **Wood and Holzapfel, (1995)** described lactic acid bacteria as Gram-positive, non-spore forming, catalase-negative cocci or rods that are anaerobic, micro-aerophilic or aero-tolerant. Lactobacilli are Gram-positive, catalase-negative, non-sporing rods (whose cell length varies between 1.5 µm and 5 µm. The cell may be slender, curved or bend when viewed under the microscope and may be in chains or network **(Edema and Sanni, 2006; 2008)**. The shape of isolates under the light microscope were short rods in clusters, short rods, long rods in network, long rods and cocci. The isolates were non-spore formers and indole negative. This imply they are unable to hydrolyze tryptophan in producing indole. The isolates were unable to utilize urea and nitrate while some produced nitrogen gas. Homofermentative organisms produces only lactic acid from glucose, examples include *L. lactis* subsp *lactis, L. lactis subsp diacetylactis and L. cremoris* while the Heterofermentative produces lactic acid, carbondioxide and aroma compounds as product of fermentation, examples include *Leuconostoc mesenteroides, Lactobacillus spp* **(Sanni et al., 1998)**. Many of the isolates had ability to produced gas from glucose. The isolates thrived at 15 ^{o}C and above. This current findings agrees with that of **Hutkins, (2006)** describing most bacteria isolates in sourdough as mesophilic, with some genuses having some species that are psychrotrophic, thermoduric or thermophilic. Some species are salt tolerant, acid tolerance, growing below pH 4.0. Lactic acid bacteria grows at an optimum pH of 5.5 - 6.5. Some strains are reported to be ethanol tolerant, bile-tolerant, aero-tolerant and some requiring strict anaerobic conditions **(Petra et al., 2011)**.

The carbohydrate fermentation pattern of presumed lactic acid bacteria isolated from sourdough is presented in Table 2. The result showed that *Lb. pentosus* and *Lb. plantarum* was most dominant cultures representing about twenty-five percent (25%), *P. pentosaseus*, *Micrococcus varians*, *Lb. buchneri* and *Lb amylovorus* (12.5%) each while *Lb. plantarum* and *Lb. pentosus* was the most dominant LAB in finger millet sourdough. The genera *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc* have been reported important in food technology **(Harrigan and McCane, 1976)**. The carbohydrate fermentation pattern of presumed yeasts isolates from pearl and finger millet sourdoughs is presented in Table 3. Results showed the dominance of *Saccharomyces cerevisiae*. *Saccharomyces cerevisiae* had weak reaction to mannitol, sorbose, sorbitol and esculin while *Candida quercitrusa* and *C. milleri* had weak reaction to trehalose and 2-keto-gluconate. Several yeast species has been reported in sourdoughs by various authors (**Hammes et al., 2004; Rossi, 1996; De Vuyst and Neysens, 2005)**. The observed lactic acid bacteria and yeast isolates agrees with those observed in previous similar works. **Jakobsen and Lei (2004); Achi (2005) and Kalui et al. (2009)** reported the occurrence of *L. plantarum, L. casei, L.sakei, L. acidophilus* and *L. salivarius* in cereal based fermented food. Also, **Kalui et al. (2009)** reported the isolation of *L. fermentum, P. pentosaceus, L. plantarum, W. confusus* and *L. rhamnosus* from *ikii*- a traditional fermented

Table 1 Morphological and biochemical characteristics of dominant presumed lactic acid bacteria and yeasts isolates from spontaneously developed pearl and finger millet sourdoughs

Isolates/Description	Colony surface	Pigment	Shape	Cell morphology	Arrangement	Motility	Catalase	Gram reaction	Spore	Urease	Nitrate	Indole	Homo/Hetero	Growth at 10 °C	Growth at 15 °C	Growth at 30 °C	Growth at 37 °C	Growth at 45 °C	Growth at 2% NaCl	Growth at 4% NaCl	Growth at 6.5% NaCl	pH 4.5	pH 6.0	Probable organism
P1	Sm	Wh	Cir	Rods	SS	-	-	+	-	-	-NG	-	Ho	-	-	+	nd	-	+	-	-	+	+	***Lactobacillus pentosus***
P2	Sm	Wh	Cir	Cocci	CL	-	-	+	-	-	-NG	-	Het	-	+	+	nd	-	+	+	-	+	+	***Pediococus pentosaseus***
P3	W	Wh	Irreg	Rods	SL	-	-	+	-	-	±G	-	Het	-	-	+	nd	+	-	+	+	+	±	***Lactobacillus plantarum***
P4	W	C	Irreg	Rods	SL	-	-	+	-	-	±G	-	Het	-	+	+	nd	+	+	+	-	+	±	***Lactobacillus buchneri***
P5	Sm	Wh	Cir	Cocci	CL	-	-	+	-	-	-NG	-	Het	-	+	+	nd	-	+	+	-	+	-	***Micrococcus varians***
P6	Sm	Wh	Cir	Rods	SC	-	-	+	-	-	-NG	-	Ho	-	-	+	nd	+	+	+	-	+	±	***Lactobacillus amylovorus***
F1	Sm	Wh	Cir	Rods	SS	-	-	+	-	-	-NG	-	Ho	-	-	+	nd	-	+	+	-	+	+	***Lactobacillus pentosus***
F2	W	C	Irreg	Rods	SL	-	-	+	-	-	+G	-	Het	-	+	+	nd	+	+	+	-	+	±	***Lactobacillus plantarum*****1**
F3	Sm	C	Cir	Rods	SS	-	-	+	-	±	+G	-	Ho	-	+	+	nd	-	+	+	+	+	+	***Lactobacillus farciminis***
F4	Sm	C	Cir	Rods	SS	-	-	+	-	±	+G	-	Ho	-	+	+	nd	-	+	+	+	+	+	***Lactobacillus farciminis***
F5	Sm	Wh	Cir	Rods	SC	-	-	+	-	-	-NG	-	Ho	-	-	+	nd	+	+	+	-	+	±	***Lactobacillus amylovorus***
F6	W	Wh	Irreg	Rods	SL	-	-	+	-	-	±G	-	Het	-	-	+	nd	+	-	+	+	+	±	***Lactobacillus plantarum***
F7	Sm	Wh	Irreg	Rods	SC	-	-	+	-	-	-NG	-	Het	-	+	+	nd	-	+	+	+	-	-	***Lactobacillus alimentarius***
F8	R	Wh	Cir	Cocci	CL	-	-	+	-	-	-NG	-	Het	-	+	+	nd	+	-	-	-	+	+	***Pediococcus pentoaseus* 1**
F9	Sm	Wh	Cir	Rods	SS	-	-	+	-	-	-NG	-	Ho	-	-	+	nd	+	-	-	-	+	+	***Lactobacillus crispatus***
PY1	Sm	C	Cir	Oval	EL	-	+	+	-	+	nd	nd	nd	nd	nd	nd	+	nd	+	+	+	nd	nd	***Saccharomyces cerevisiae***
PY2	Sm	C	Cir	Oval	EL	-	+	+	-	+	nd	nd	nd	nd	nd	nd	+	nd	+	+	+	nd	nd	***Candida quercitrusa***
PY3	Sm	C	Cir	Oval	EL	-	+	+	-	+	nd	nd	nd	nd	nd	nd	+	nd	+	+	+	nd	nd	***Candida milleri***
PY4	Sm	C	Cir	Oval	EL	-	+	+	-	+	nd	nd	nd	nd	nd	nd	+	nd	+	+	+	nd	nd	***Saccharomyces cerevisiae***
FY1	Sm	C	Cir	Oval	EL	-	+	+	-	+	nd	nd	nd	nd	nd	nd	+	nd	+	+	+	nd	nd	***Saccharomyces cerevisiae***
FY2	Sm	C	Cir	Oval	EL	-	+	+	-	+	nd	nd	nd	nd	nd	nd	+	nd	+	+	+	nd	nd	***Saccharomyces cerevisiae***
FY3	Sm	C	Cir	Oval	EL	-	+	+	-	+	nd	nd	nd	nd	nd	nd	+	nd	+	+	+	nd	nd	***Saccharomyces cerevisiae***

Key: + = positive reaction, - = negative reaction, ± = Weak reaction, +G = Positive and gas production, -NG = Negative and no gas production, ±G = Weak reaction and gas production, **Sm= Smooth, W= wrinkled, R= Rough, Cir = Circular, Wh = White, C= Creamy, Irreg = Irregular, SS = Short and Singly, CL= Clusters, SL=Short and long, SC= Short and in Clusters, EL = Ellipsoidal, nd = not determined.**

Table 2 The carbohydrate fermentation pattern of dominant presumed lactic acid bacteria isolates obtained from spontaneously developed pearl and finger millet sourdoughs

Isolates/Description	Glucose	Sucrose	Maltose	D-xylose	Sorbitol	Glycerol	Starch	Arabinose	Mannitol	Galactose	Raffinose	Esculin	Lactose	Trehalose	Melebiose	Inositol	Melezitose	α-methyl-mannoside	Salisin	Ribose	Cellobiose	Inulin	Fructose	Dextrose	Mannose	Rhamnose	Xylose	Sorbose	Malonate	Citrate	Glucosamine	Dulcitol	Adonitol	α-methyl-glucoside	Xylitol	ONPG	Saccharose	Probable Microorganism
P1	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	-	V	-	+	***Lb. pentosus***
P2		+	-	**V**	-	-	-	+	-	+	-	+	**V**	+	**V**	-	-	-	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	+	***P. pentosaseus***
P3	+	+	+	+	+	-	-	-	-	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	***Lb. plantarum***
P4	+	+	+	+	-	-	-	+	-	**V**	-	**V**	**V**	-	+	+	-	-	-	+	-	+	+	+	-	-	**V**	-	-	-	-	-	-	-	-	-	-	***Lb. buchneri***
P5	+	+	+	+	-	-	-	-	**V**	-	-	-	**V**	**V**	**V**	-	-	-	+	+	-	-	**V**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	***M. varians***
P6	+	+	+	+	-	+	+	-	-	+	-	+	-	+	-	-	**V**	-	+	-	+	+	+	+	+	-	-	-	+	-	-	-	-	-	**V**	-	+	***Lb. amylovorus***
F1	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	-	**V**	-	+	***Lb. pentosus***
F2	+	+	+	+	+	-	-	-	-	**V**	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	**V**	-	-	-	-	-	-	-	-	-	-	+	***Lb. plantarum* 1**
F3	+	+	+	**V**	-	-	-	-	-	+	-	+	+	+	-	-	-	-	+	-	+	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	+	***Lb. farciminis***
F4	+	+	+	**V**	-	-	-	-	-	+	-	+	+	+	-	-	-	-	+	-	+	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	+	***Lb. farciminis***
F5	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	**V**	-	+	+	+	+	+	+	+	-	+	-	**V**	-	-	-	-	-	**V**	-	+	***Lb. amylovorus***
F6	+	+	+	+	+	-	-	-	-	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	***Lb. plantarum***
F7	+	+	+	+	-	-	-	**V**	-	+	-	+	-	+	-	+	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	***Lb. alimentarius***
F8	+	+	-	**V**	-	-	-	+	-	+	-	+	**V**	+	**V**	-	-	-	+	+	+	-	+	-	+	**V**	-	-	+	-	-	-	-	-	-	-	**V**	***P. pentoaseus* 1**
F9	+	+	+	+	-	+	-	-	+	+	-	+	+	-	-	-	-	-	+	-	+	+	+	+	+	-	-	-	+	-	-	-	-	-	**V**	-	+	***Lb. crispatus***

Keys: + = Positive reaction, - = Negative reaction, **V**= weak reaction,

Table 3 The carbohydrate fermentation pattern of dominant presumed yeasts isolates obtained from spontaneously developed pearl and finger millet sourdoughs

Isolates/Description	Glucose	Maltose	Mannitol	D-xylose	Sorbitol	Glycerol	Actidione	L-arabinose	Mannitol	Galactose	Raffinose	Esculin	Lactose	Trehalose	Melebiose	Inositol	Melezitose	Ribose	Cellobiose	Rhamnose	D-Xylose	Sorbose	Erythritol	N- acetyl glucosamine	α-methyl-glucoside	Saccharose	DL-lactate	2-keto-gluconate	Palatinose	Glucoronate	Gluconate	Levulinate	Glucosamine	Probable microorganism
PY1	+	-	**V**	+	**V**	-	-	-	**V**	+	+	**V**	-	-	-	-	-	-	-	-	-	**V**	-	-	**V**	+	-	**V**	-	-	-	-	-	***S. cerevisiae***
PY2	+	+	+	+	+	+	-	-	+	+	-	-	-	+	-	-	-	+	-	-	**V**	+	-	+	-	+	-	+	+	-	+	+	+	***C. quercitrusa***
PY3	+	-	+	+	+	+	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	***C. milleri***
PY4	+	-	**V**	+	**V**	-	-	-	+	+	+	**V**	-	**V**	-	-	-	-	-	-	-	**V**	-	-	**V**	+	-	**V**	-	-	-	-	-	***S. cerevisiae***
FY1	+	-	**V**	+	**V**	-	-	-	**V**	+	+	**V**	-	-	-	-	-	-	-	-	-	**V**	-	-	**V**	+	-	**V**	-	-	-	-	-	***S. cerevisiae***
FY2	+	-	**V**	+	**V**	-	-	-	+	+	+	**V**	-	**V**	-	-	-	-	-	-	-	**V**	-	-	**V**	+	-	**V**	-	-	-	-	-	***S. cerevisiae***
FY3	+	-	**V**	+	**V**	-	-	-	+	+	+	**V**	-	**V**	-	-	-	-	-	-	-	**V**	-	-	**V**	+	-	**V**	-	-	-	-	-	***S. cerevisiae***

Keys: + = Positive reaction, - = Negative reaction, **V**= weak reaction.

maize porridge in Kenya. The number and type of yeast species found in sourdoughs depends on several factors including the degree of dough hydration and type of cereal used. The dominance of *S. cerevisiae* might be due to its ability to utilise simple sugar in the cereals faster during fermentation.

Fig. 2 and 3 presents the mean pH, temperature and titratable acidity of spontaneously developed sourdoughs from pearl and finger millet. From Figure 2 and figure 3, the pH of the spontaneously developed sourdoughs decreased with increasing days of fermentation. The pH ranged from 4.9 to 3.5 in pearl millet sourdough while in finger millet sourdough it ranged from 5.7 to 3.3. This agrees with results obtained by **Sanni et al. (1998)** in a work on sour maize bread production using starter cultures. The significant decrease in pH might be due to increase acid production by lactic acid bacteria in the sourdough. No significant difference was observed in the pH of the pearl millet sourdoughs on the 6th and 7th day of fermentation.

The temperature of the spontaneously developed pearl and finger millet sourdoughs increased with increasing days of fermentation. This might be due to increased rate of reaction in the sourdough as metabolites production increased. Temperature increases from 27 ^{o}C to 32 °C in sour maize bread produced by **Sanni et al. (1998)** however, after 48 h of fermentation the temperature was constant at 32 °C and the pH reduced further to 3.7. **Hounhouigan et al. (1993)** reported titratable acidities ranging from 1.2 - 1.4 in `mawe', a fermented maize dough. Titratable acidities increased significantly with increasing days of fermentation. Titratable acidity ranged from 1.5 ml to 3.4 ml and 1.1 ml to 3.6 ml in spontaneously developed pearl and finger millet sourdoughs. The dominant metabolite of lactic fermentation is lactic acid although acetic acid also contributes to acidification **(Petra et al., 2011)**. Acetic acid is important for strong aroma, fungicidal and antimicrobial effect in fermented products while lactic acid influences product texture **(Corsetti and Settanni, 2007).**

Fig 4 and 5 presents the microbial population of mesophilic bacteria and fungi of spontaneously developed pearl and finger millet sourdoughs. The mesophilic bacterial count increased with increasing fermentation days. The bacteria count of pearl and finger millet sourdough ranged from 53 x 10 ^{8} - 280 x 10 ^{8} Cfu/ml and 28 x10 ^{9} cfu/ml - 50 x10 ^{9} cfu/ml respectively. The bacterial count of developed finger millet sourdough was highest on day 4 of fermentation. The fungi growth increased with increasing days of fermentation. The growth pattern of associated microorganisms followed a normal bacterial growth curve. This could be that the microbes were still in an adaptation phase (Lag phase) between the first two days of fermentation. At day 2 and 3 the mesophilic bacterial count increased (exponential phase) which might be due to the activity of the microbes in adjusting to the new environment of fermentation. At day 4 and 5, the mesophilic bacterial count declined, this might be due to accumulation of metabolites, increased competition for limited nutrient resulting in death of the microorganisms. The bacteria involved in sourdough fermentations are mainly mesophilic **(De-vuyst and Neysens, 2005)**. The microflora population of cereals flours ranged from 2x10^{4}–6x10^{6} CFU/g **(Stolz, 1999)**. The occurrence of yeasts up to 2x10^{3} CFU/g have been reported in cereals flours **(De-vuyst and Neysens, 2005)**. The values obtained in this study are slightly higher than those reported by **Stolz, (1999)**. The higher population of microbial counts might be due to pretreatment of the grains (fermentation).

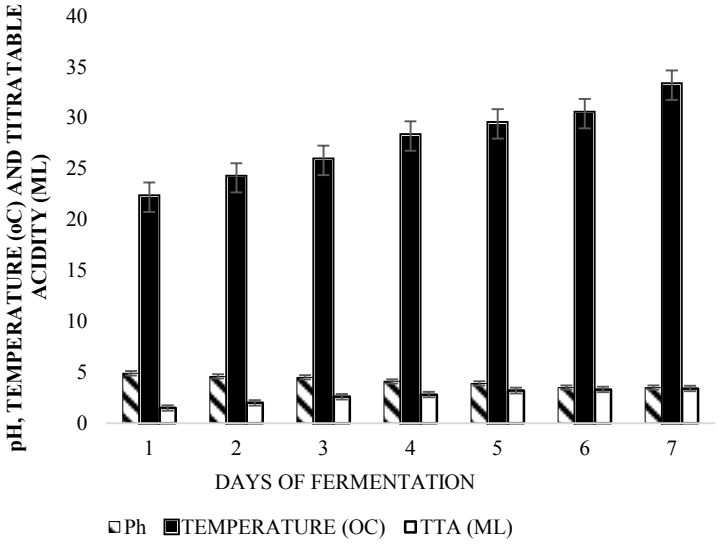


Figure 2 pH, Temperature (^{o}C) and Titratable acidity (ml) of spontaneously developed sourdough from Pearl millet flour

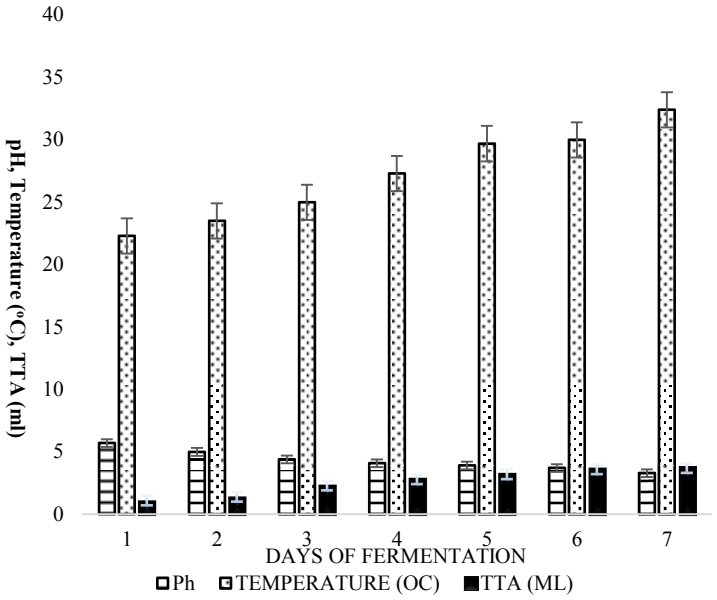


Figure 3 pH, Temperature (^{o}C) and Titratable acidity (ml) of spontaneously developed sourdough from Finger millet flour

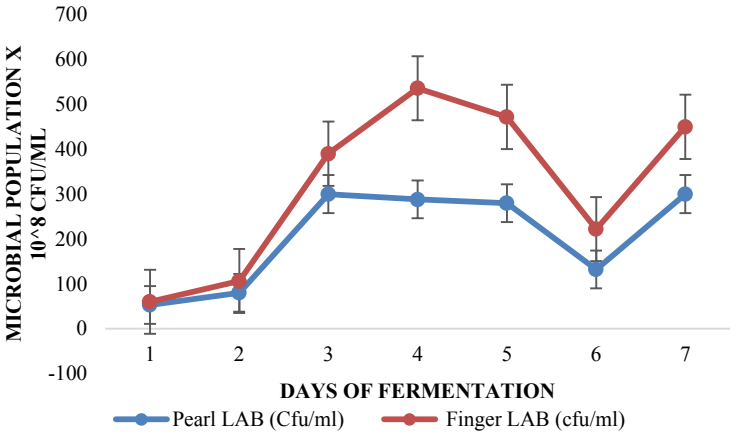


Figure 4 The microbial population of LAB isolated from spontaneously developed sourdough using pearl and finger millet flour

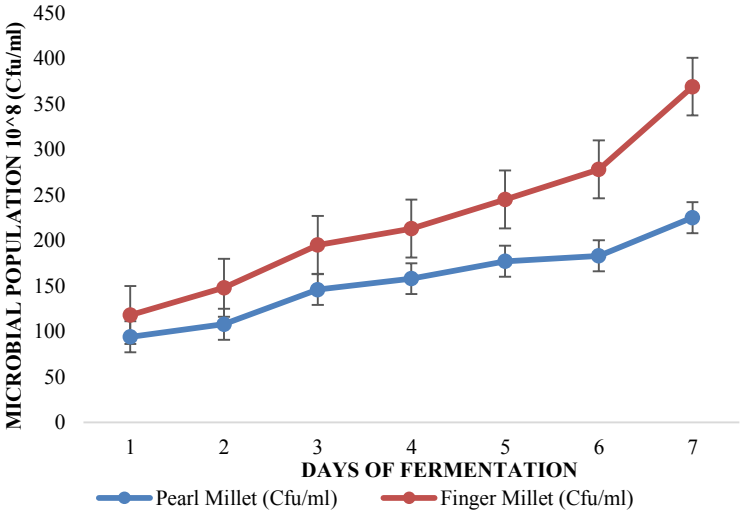


Figure 5 The microbial population of yeast isolated from spontaneously developed finger and pearl millet flour sourdough

CONCLUSION

Pearl and finger millet flour are rich sources of beneficial microbial consortia especially LAB and yeast. The titratable acidity, pH, temperatures of fermenting millet meals increased with increasing days of fermentation. Spontaneously developed pearl millet sourdoughs were dominated by *Lb. plantarum* (Heterofermenters) and *Lb. pentosus*, *P. pentosaseus* (Homofermenters) while

spontaneously fermented finger millet sourdoughs is dominated by *Lb. plantarum* 1, *Lb. pentosus* and *P. pentosaseus*. *S. cerevisiae* and *Candida milleri* are dominant yeast cultures in millet sourdoughs. Further studies should be done on the characterisation of microbial isolates using molecular techniques and screening of isolated cultures for desirable sourdough functional properties.

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AMINO ACIDS PRODUCTION BY PROTEOLYTIC *MUCOR MUCEDO* STRAIN SEE1 ON THE OPTIMIZED FERMENTATION MEDIUM

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ABSTRACT

The present study has been conducted to isolate the potential fungal isolates in proteolysis process. One isolate showed high activity in protease production, being *Mucor* sp. Morphological and microscopical studies, as well as 18S rRNA sequence confirmed that this isolate is identified as *Mucor mucedo* strain SEE1 and sequencing product was deposited in the GenBank database under the accession number KP736529. Optimization of protease production during Plackett-Burman and central composite designs has been carried out, with six nutritional variables. Glucose and casein were significantly superior to other variables in protease production. Moreover, an overall 3.36-fold increase in protease production has been obtained by Placket-Burman. However, 1.14-fold increase was found in central composite design. On the other hand, during the fermentation process in optimized medium, liberated amino acids has been determined (µg/ml), where histidine came in the first order (1155.3) followed by lysine (897.4), tyrosine (634), arginine (580) and isoleucine (580), respectively. However, cysteine was found in less appreciable amount (36).

Keywords: Amino acids production; *Mucor mucedo* strain SEE1; protease; statistical optimization; 18S rRNA

INTRODUCTION

Proteolysis process occurred through the chemical or enzymatic hydrolysis of proteinaceous compounds, several microbial enzymes are involved in this process. In this respect, protease enzymes are one of the most important microbial enzymes, which widely used in many industries, e.g. food, pharmaceutical, detergent, leather and silk **(Gessesse, 1997, Kembhavi *et al.*, 1993, Ivanov *et al.*, 2013)**.This group represents one of the largest groups of industrial enzymes and accounts for approximately 60% of the total enzyme sales in the world **(Zambare *et al.*, 2011)**. Moreover, microbial proteases are gaining more importance than conventional chemicals that cleave protein, produce peptides and amino acids because of the cheaper production cost and use of renewable resources **(Souza *et al.*, 2015)**. These enzymes can be produced from bacteria, fungi and yeasts using many processes like submerged fermentation culture **(Haki and Rakshit, 2003)**. Currently, a large proportion of commercially available proteases is derived from fungi such as *Aspergillus flavus* **(Kranthi *et al.*, 2012)**. *Aspergillus oryzae* **(Vishwanathaet *al.*, 2010a)**, *Penicillium griseoroseum* **(Ikram-Ui-Haq and Mukhtar, 2007)**, *Rhizopus oryzae* **(Kumar *et al.*, 2005)**, *Mucor circinelloides* **(Andrade *et al.*, 2002)** and *Thermomucor indicaeseudaticae* **(Merheb-Dini *et al.*, 2010)**. Although, fungal proteases have attracted the attention of environmental biotechnologists because fungi can grow on low-cost substrates and secrete large amount of enzymes intotheculture medium, which could ease downstream processing **(Anitha and Palanivelu, 2013)**. As well as, the production cost acting 30-40% of the growth medium and this is interest from an industrial point of view **(Joo *et al.*, 2003)**. Generally, protease production is greatly influenced by media component and physical factors **(Potumarthi *et al.*, 2007)**. Wherein, the industrial fermentation is moving away from traditional and largely empirical operation towards knowledge based and better-controlled process **(Singh *et al.*, 2004)**. Therefore, a number of optimization techniques could be used for this purpose, by which the statistical approaches offer ideal ways for optimization process in biotechnology **(Gupta *et al.*, 2002)**. Response surface methodology one of these approaches, which includes center composite design, helps in evaluating the effective factors and in building models to study interaction and select optimum conditions of variables for a desirable response **(Dutta *et al.*, 2004)**. Proteolysis process generally associated with some amino acids and nitrogenous compounds **(Ventura et al., 2012)**. Amino acids have been taken as an important tool for nutritional status, especially in patients suffering from inborn errors of metabolism (IEM) and for monitoring therapy in patients with IEM, as well. Additionally, amino acids are recommended as food supplements for body building, sleep aid, depression therapy, premenstrual dysphoric disorder, smoking cessation, bruxism **(Ivanov *et al.*, 2013)**. Wherein, using the new biotechnology for such production of these amino acids considered a new trend to face the shortage in human nutrition, especially in developed countries. Herein, our study aimed to (i) study the efficacy of the new isolate of *Mucor* sp. in proteolysis and optimization of protease enzyme, (ii) determination of amino acids associated with the proteolysis process.

MATERIAL AND METHODS

Mucor sp. was isolated on skim agar plates. The fungus showed high proteolytic activity among all the isolated ones, so it was selected for the next trials. The selected fungus was identified upon the morphological and microscopic investigations, according to **Domsch *et al.* (1980)**, in addition to molecular identification.

Molecular identification of the isolated *Mucor* sp.

A 18S rRNA sequencing was performed by Macrogen Korea Company Gasan-dong, Geumchen-gu, Seoul, Korea (http://www.macrogen.com**).** The genomic DNA was isolated by transferring fungal mycelium with a sterilized toothpick, suspended in 0.5 ml of sterilizes saline in a 1.5 ml centrifuge tube and centrifuged at 10000 rpm for 10 min. Then the supernatant was discarded and the pellet was resuspended in 0.5 ml of InstaGene Matrix (Bio-Rad, USA). Incubated at 56 °C for 30 min and then heated at 100 °C for 10 min. After heating, the supernatant can be used for PCR. The PCR amplification reaction was performed in a total volume of 100 µl, which contained 1 µl DNA, 10 µl of 250 mM deoxyribonucleotide 5'-triphosphate (dNTP's); 10 µl PCR buffer, 3.5 µl 25 mM $MgCl_2$ and 0.5 µl Taq polymerase, 4 µl of 10 pmol (each) forward primer ITS1 (3'-TCCGTAGGTGAACCTGCGG-5') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and water was added up to 100 µl. The PCR-apparatus was programmed as follows: 5 min denaturation at 94 °C,

followed by 35 amplification cycles of 1 min at 94 °C, 1 min of annealing at 55°C, and 2 min of extension at 72 °C, followed by a 10 min final extension at 72 °C. Unincorporated PCR primers and dNTPs were removed from PCR products by using Montage PCR Cleanup kit (Millipore). The PCR reaction mixture was then analyzed via 1% (w/v) agarose gel electrophoresis (Figure1), and the remaining mixture was purified using QIA quick PCR purification reagents (Qiagen, USA).

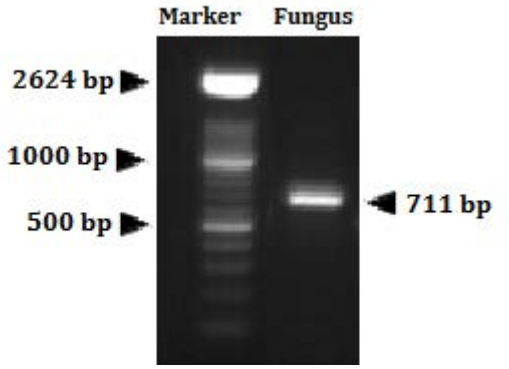

Figure1 Agarose gel electrophoresis of the amplified ITS fragment of the *Mucor mucedo* strain SEE1.

The purified PCR products were sequenced. Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using an ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq-DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the last mentioned PCR-primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). The sequencing product was deposited in the GenBank database under accession numbers KP736529.The 18S rRNA gene sequence (667bp) of the strain was aligned with the corresponding 18S rRNA sequences of the type strains of representative members of the fungi retrieved from the GenBank, EMBL, DDBJ and PDB databases by using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) **(Altschul *et al*., 1997)** and the software package MEGA4 version 2.1 **(Tamura *et al*., 2007)** was used for multiple alignment and phylogenetic analysis. The phylogenetic tree was constructed via the neighbor-joining algorithm **(Saitou and Nei, 1987)** based on the 18S rRNA gene sequences of the strain and related organisms.

Submerged liquid state fermentation (LSF) and time course profile

Plates of Sabouraud agar were inoculated and incubated at 35 °C for 72 h, the inoculum was obtained by scraping the agar surface in the presence of sterile water, to obtain 10^6spore ml^{-1}, by Neubauer Chamber. The composition of the protease production medium contained (g l^{-1}), glucose (18), peptone (8), casein (4), KH_2PO_4 (2), olive cake (4) and corn steep liquor (CSL) (4). The initial pH was adjusted to 5.0 and sterilized at 121°C for 15 minutes. 10 % (v/v) of inoculum was transferred to 250-ml Erlenmeyer flasks containing 45 ml of sterilized liquid fermentation medium. Incubation was carried out at 35 °C under shaking at 150 rpm. The optimum incubation period was determined on this medium along for 5 days.

Protease activity assay

Protease activity was quantitatively assayed in a mixture consisted of 1 ml crude protease and 1 ml of 2 % casein in 0.1M tris-HCL buffer, pH 7.5, then incubated at 37°C for 20 min **(Abou-Ayana *et al*., 2015)**. One unit (U) of protease activity was defined as the amount of the enzyme resulting in the release of 0.5 µg of tyrosine equivalent $ml^{-1}min^{-1}$ under the assay conditions.

The fractional factorial Plackett-Burman design

Six components of LSF medium were screened at high (+1) and low (-1) levels in addition to three center point (0), using Plackett-Burman design. The relation between the coded and actual values was calculated by the following equation:

$$x_i = (X_i - X_0)/\Delta X_i$$

Where; x_i is the coded value of an independent variable, Xi is the real value of an independent variable, X_0 is the real value of an independent variable at the center point, and ΔX_i is the step change value. The main effect of each variable was calculated using the following equation:

$$\text{Effect } (\times_i) = 2\left(\sum M_i^{+1} - M_i^{-1}\right)/N$$

Where; X_i is the effect of the tested variable. M_i^{+1} and M_i^{-1} represent fungal protease production from the trials where the variable (X_i) measured was present at high and low concentration, respectively, N, the total number of trials.

Central composite design

Fermentation factors affecting fungal protease production were optimized using the full central composite design (CCD). The significant variables (Glucose, X_1 and casein, X_3) from screening experiment were further investigated for studying the interaction between the two variables. The other medium components were kept at their minimal concentrations. Each of the two factors was examined at five different levels; at the center point and an axial point located at a specified distance (alpha, $\alpha = 1.414$) from the design center in each direction on each axis. According to the applied design, 13 combinations were executed**.** The observations of the two factors were fitted to the following second order polynomial quadratic model:

$$Y = \beta_0 + \sum\beta_i X_i + \sum\beta_{ij} X_i X_j + \beta_{ii} X_i^2$$

Where; Y is the predicted response, β_0 model constant, X_i, and X_j independent variables; β_i, is linear coefficients; β_{ij}, is cross product coefficients and β_{ii} is the quadratic coefficients.

Statistical analysis

The statistical analysis of Plackett-Burman and CCD results was performed with the aid of statistical analysis software Minitab ver. 10. The statistical analysis software package, Statistica ver. 8.0, was used to create the three-dimensional surface plot.

Amino acids determination

Free amino acids were quantified from culture supernatants. The culture samples were filtered and hydrolyzed with 6 M HCL for 24h at 110 °C under a vacuum, and amino acid contents were measured using Sykam57130amino acid reagent organizer **(Lee *et al*., 2014)**.

RESULTS AND DISCUSSION

Fungal isolates were selected according to their proteolysis activity on skim milk agar, the potent isolate was selected and morphologically investigated. This isolate is belonging to *Mucor* sp. These findings are in accordance with previous studies that showed a great number of fungal strains have potential proteolysis, e.g. *Aspergillus niger* **(O'Donnell *et al*., 2001)**, *A. flavus* **(Kranthi *et al*., 2012)**, *Mucor pusillus*, *M. miehei* and *M. circinelloides* **(Andrade *et al*., 2002)**.

Molecular identification

The nucleotide sequence of *Mucor mucedo* strain SEE1 has been compared with other fungal species sequences using BLAST algorithm at the website http://blast.ncbi.nlm.nih.gov/Blast.cgi, and the sequence was assembled and deposited in the NCBI Genbank with accession number KP736529. The phylogenetic analysis of *Mucor mucedo* strain SEE1revealed 100% similarity with *Mucor mucedo* strain CBS 987.68 (accession number JN939204.1). A phylogenetic tree (Figure2) was constructed according to the neighbor-joining method of **Saitou and Nei (1987)** with MEGA4 **(Tamura *et al.*, 2007)**. The topology of the phylogenetic tree was evaluated by using the bootstrap resembling method of **Felsenstein (1985)** with 1000 replicates. This tree showed a close phylogenetic association of *Mucor mucedo* strain SEE1 with *M. mucedo* species. Phylogenetic analysis indicated that the strain SEE1 falls into a clade together with *M. mucedo* strain CBS 640.67 (accession number HM849687.1, 99% similarity to *M. mucedo* strain SEE1), *M. piriformis* strain CBS 169.25 (accession number HM849681.1, 95% similarity to *Mucor mucedo* strain SEE1),*M. luteus* strain CBS 243.35 (accession number HM849685.1, 93% similarity to *M. mucedo* strain SEE1), *Rhizomucor variabilis* strain CBS 103.93 (accession number HM849684.1, 93% similarity to *M. mucedo* strain SEE1).Therefore, it is proposed that strain SEE1should be included in the genus *Mucor* as *M. mucedo* strain SEE1.

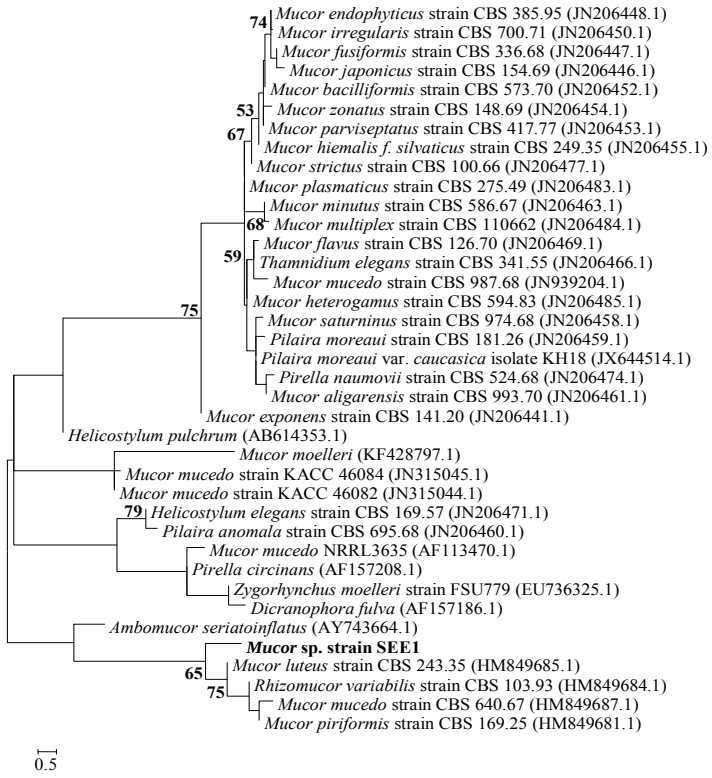

Figure 2 The phylogenetic tree of *M. mucedo* strain SEE1 with respect to the closely related sequences available at the GenBank.

Time course for protease production

Time course of protease production by *M. mucedo* strain SEE1 was monitored for 5.0 days at 24 hr intervals. As shown in Figure3, the maximum protease production was recorded after the second day of fermentation (3.859U/ml/min). Then, protease production is decreased with further time of incubation.

Screening of medium components by Plackett-Burman design

The influence of six variables, namely; glucose, peptone, casein, KH_2PO_4, olive cake and CSL, on the protease production by *M. mucedo* strain SEE1 was investigated using Plackett-Burman design with three center points. Among the screened medium component, several nitrogen sources were evaluated to select the suitable one for amino acids biosynthesis, of them CSL as an economic source of nitrogen may also act as a carbon source at the same time, however, olive cake may be served as another source of carbon.The design and the corresponding response of protease production are shown in Table (1).

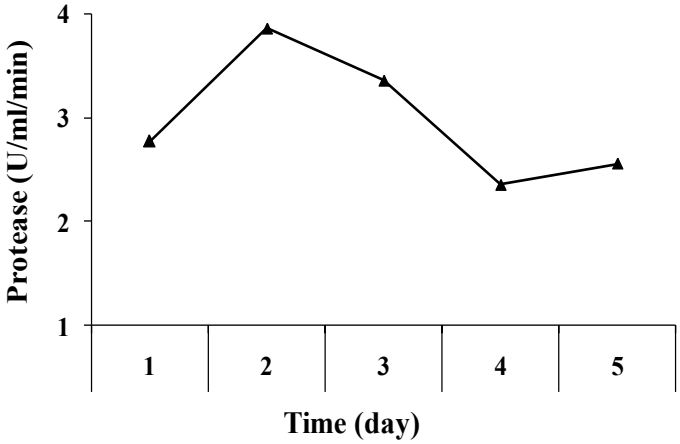

Figure3 Profile of protease production by *M. mucedo* strain SEE1 along 5 days incubation

Table 1 Actual and coded values of medium components based on Plackett-Burman design and corresponding experimental and fitted protease activity by *M. mucedo* strain SEE1.

Run	Medium component (g/l)						Protease (U/ml/min)		Residual
	Glucose (X_1)	Peptone (X_2)	Casein (X_3)	KH_2PO_4 (X_4)	Olive cake (X_5)	CSL (X_6)	Experimental	Fitted	
1	25 (1)	4 (-1)	6 (1)	1 (-1)	2 (-1)	2 (-1)	11.600	10.332	1.268
2	25 (1)	12 (1)	2 (-1)	3 (1)	2 (-1)	2 (-1)	9.133	9.657	-0.524
3	11 (-1)	12 (1)	6 (1)	1 (-1)	6 (1)	2 (-1)	8.550	8.157	0.393
4	25 (1)	4 (-1)	6 (1)	3 (1)	2 (-1)	6 (1)	12.967	12.988	-0.021
5	25 (1)	12 (1)	2 (-1)	3 (1)	6 (1)	2 (-1)	10.383	9.859	0.524
6	25 (1)	12 (1)	6 (1)	1 (-1)	6 (1)	6 (1)	11.467	11.657	-0.19
7	11 (-1)	12 (1)	6 (1)	3 (1)	2 (-1)	6 (1)	10.300	10.61	-0.31
8	11 (-1)	4 (-1)	6 (1)	3 (1)	6 (1)	2 (-1)	8.550	9.69	-1.14
9	11 (-1)	4 (-1)	2 (-1)	3 (1)	6 (1)	6 (1)	10.900	9.429	1.471
10	25 (1)	4 (-1)	2 (-1)	1 (-1)	6 (1)	6 (1)	9.217	10.274	-1.057
11	11 (-1)	12 (1)	2 (-1)	1 (-1)	2 (-1)	6 (1)	7.800	7.693	0.107
12	11 (-1)	4 (-1)	2 (-1)	1 (-1)	2 (-1)	2 (-1)	6.050	6.571	-0.521
13	18 (0)	8 (0)	4 (0)	2 (0)	4 (0)	4 (0)	3.550	3.783	-0.233
14	18 (0)	8 (0)	4 (0)	2 (0)	4 (0)	4 (0)	4.100	3.783	0.317
15	18 (0)	8 (0)	4 (0)	2 (0)	4 (0)	4 (0)	3.700	3.783	-0.083

Number between parentheses is the corresponding coded value

The protease production varied intherange of 3.550 (in run no. 13) to 12.967 U/ml/min (run no. 4), this variation reflects the importance of medium optimization to attain higher productivity. Additionally, the low values of the residuals reflect the adequacy and accuracy of the design. The statistical analysis of Plackett-Burman design was performed (Table 2).

The analysis of variance (ANOVA) demonstrated that the model was highly significant as was evident from the Fisher's *F*-test with a very low probability value (*P*-value*)* =0.001). The model *F*-value of 15.76 implies the model is significant. The higher *F*-value and the lower *P*-value, the more significant of the model. Models with *P*-values of less than 0.05 were considered to be significant. The *P*-value of the lack of fit error is another important parameter to evaluate the model, this *P*-value (0.053) did not reach the level of significant. The lack of fit is an indication for the appropriateness of the data, as fitting of the model is required and reflects a good parameter. To check the goodness of fit of the model, the determination coefficient (R^2) was estimated. The R^2 values provide a measure of how much variability of the observed response can be explained by the experimental factors. The R^2 value is always between 0 and 1. The closer the R^2 value to 1, the stronger the model is, and the better it predicts the response. In our study, the value of R^2 = 94.03% indicated that 94.03 % of the variability in the response could be explained by the model and only 5.97% of the total variations are not explained by the model, which may be back to the error. In addition, the value of the adjusted (adj.) R^2 is high enough to emphasize the accuracy of the relationships between the studied variables and protease production by the isolated fungus.

Table 2 Analysis of variance of the Plackett-Burman designf or the overall model as well as each variable affecting protease production by *M. mucedo* strain SEE1.

Source		Degree of freedom	Contribution (%)	Adj Sum of squares	*F*-value	*P*-value	Determination coefficient (%)
Overall model	Model	7	94.03	117.72	15.76	0.001	R^2 = 94.03
	Lack of fit	5	5.84	7.31	18.08	0.053	Adj. R^2 = 88.07
	Pure error	2	0.13	0.16			
	Total error	7	5.97	7.47			
Individual	Glucose	1	10.60	13.27	12.44	0.01	
	Peptone	1	0.18	0.23	0.21	0.658	
	Casein	1	6.59	8.25	7.74	0.027	
	KH_2PO_4	1	3.79	4.75	4.45	0.073	
	Olive cake	1	0.10	0.12	0.12	0.744	
	CSL	1	4.68	5.86	5.49	0.052	

The significance of each of the tested variable was determined based on *P*-value. The statistical analysis of data obtained based on Plackett-Burman design show that, glucose with *P*-value of 0.01 was determined to be the most significant variable affecting protease production by *M. mucedo* strain SEE1 followed by casein with *P*-value of 0.027, the lower probability values ($P \leq 0.05$) of the factors indicates significant effect on protease production, the other variables of the medium components did not show any significance. The main effect of each variable on protease production was estimated (Figure 4), in which both of glucose and casein exerted significant positive effects in this respect. On the other hand, peptone exerted an insignificant negative effect. Although the other variables (KH_2PO_4, olive cake and CSL) showed positive effect, they were also out of significance. These findings are clearly presented in Pareto chart of the standardized effects (Figure 5), which allows detecting the order and significant effects of variables affecting protease production in Plackett-Burman design.

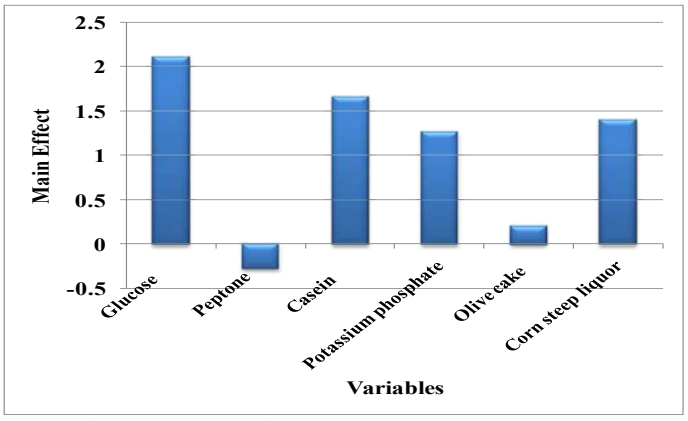

Figure4 The main effects of the fermentation medium constituents on protease production by *M. mucedo* strain SEE1 according to the Packett–Burman experimental results.

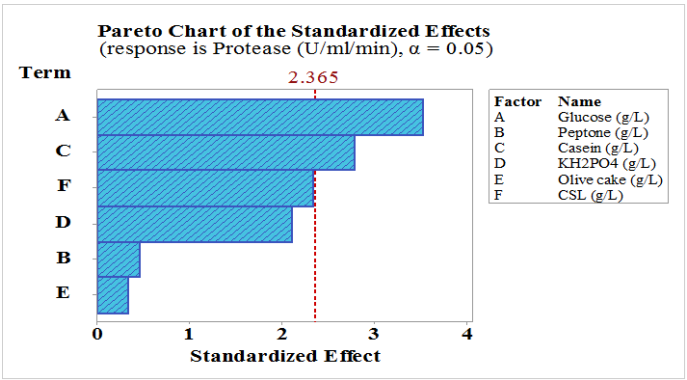

Figure 5 Pareto chart shows the order and significance of the variables affecting protease production by *M. mucedo* strain SEE1.

It demonstrates the relation between effects vs. ranks using the absolute values of the effects and draws a reference line on the chart (with a value of 2.365), which is calculated based on the data obtained from Packett–Burman experimental results. Any effect that extends past this reference line is potentially significant. As shown, glucose was the most significant variable affecting protease production at 99% confidence followed by casein at 97.30% confidence, while other variables are not significant. By neglecting the terms that were insignificant ($P > 0.05$), the first order polynomial prediction equation was derived, representing protease production as a function of the independent variables as follows:

$$Y_{(\text{Protease activity})} = 2.80 + 0.1502\ X_1 + 0.415\ X_3$$

The coefficients of glucose and casein of the previous equation were positive (0.1502 and 0.415, respectively), which means that the increase in the concentrations of these variables could exert positive effect on protease activity. Glucose and casein were chosen for further optimization using central composite design (CCD), since these factors are the only significant positive variables on protease production. Both of them were tested around the high level and the other variables that exerted insignificant effect on protease production were maintained at the low levels in the next optimization step. Data of screening of the medium components with Plackett-Burman show that run no 4 was the highest, being 12.967 U/ml/min, which is higher by about 3.4 folds obtained before applying the Plackett-Burman design.

Central composite design for optimizing protease production

Optimization was applied on the significant nutritional parameter selected from the preceding Plackett-Burman design, i.e glucose (X_1) and casein (X_3) using the central composite design (CCD) at five levels each. The other insignificant variables of the medium components were kept at their minimal concentrations. Central composite design matrix (coded and actual levels of the variables) and responses (experimental and predicted protease activity) for the 13 runs of the design are presented in Table 3.Theresponse by the fungus against the various runs of CCD shows considerable variation in protease activity. The highest levels of protease activity (14.732 U/ml/min) were achieved in runs 4 and 6; while the lowest protease activity (7.668 U/ml/min) was achieved in run number 9, where 23.5 g/l of glucose and 6 g/l of casein were used.

The statistical analysis of the data (Table 4) shows that the value of R^2 = 98.86%indicated that 98.86% of response variations was attributed to the independent variables and only 1.14% of the total variations cannot explained by the model. A regression model having an R^2 value higher than 0.9 is considered as having a very high correlation **(Chen *et al.*, 2009)**. Therefore, the present R^2-value reflected a very good fit between the observed and predicted (calculated) protease responses, and implied that the model is reliable for protease production in the present study. In addition, the value of the adj. R^2 (98.05%) was also very high to advocate for a high significance of the model. The predicted R^2 value of 94.68% was in a reasonable agreement with the adj. R^2 value. The high value of predicted R^2 indicates how well the model predicts responses for new observations that are not tested in the design, this in turn; improve the predictive ability of the model.

Table 3 Values of the independent variables used in central composite design matrix with response values of fungal protease production by *M. mucedo* strain SEE1.

Run	Codded value		Actual value (g/l)		Protease activity (U/ml/min)		Residuals
	Glucose (X_1)	Casein (X_3)	Glucose	Casein	Experimental	Fitted	
1	-1	-1	20.00	4.00	10.277	10.04	0.237
2	1	-1	27.00	4.00	11.232	10.998	0.234
3	-1	1	20.00	8.00	8.050	8.293	-0.243
4	1	1	27.00	8.00	14.732	14.979	-0.247
5	-1.414	0	18.55	6.00	9.323	9.321	0.002
6	1.414	0	28.45	6.00	14.732	14.725	0.007
7	0	-1.414	23.50	3.17	9.005	9.34	-0.335
8	0	1.414	23.50	8.83	11.264	10.92	0.344
9	0	0	23.50	6.00	7.668	8.05	-0.382
10	0	0	23.50	6.00	8.432	8.05	0.382
11	0	0	23.50	6.00	8.050	8.05	0
12	0	0	23.50	6.00	7.859	8.05	-0.191
13	0	0	23.50	6.00	8.241	8.05	0.191

Table 4 Analysis of variance and regression coefficients for protease production by *M. mucedo* strain SEE1 using central composite design.

Source	Degree of freedom	Adj Sum of squares	*F*-value	*P*-value	Regression coefficient
Model	5	71.6782	121.37	0.000	8.05
Linear	2	31.7044	134.21	0.000	
X_1	1	29.2096	247.3	0.000	1.911
X_3	1	2.4949	21.12	0.002	0.558
Square	2	31.7741	134.51	0.000	
X_1X_1	1	27.4552	232.45	0.000	1.987
X_3X_3	1	7.5249	63.71	0.000	1.04
2-Way Interaction	1	8.1996	69.42	0.000	
X_1X_3	1	8.1996	69.42	0.000	1.432
Error	7	0.8268			
Lack-of-Fit	3	0.462	1.69	0.306	
Pure Error	4	0.3648			
Total	12	72.505			

R^2 = 98.86%, adj. R^2 = 98.05%, predictedR^2 = 94.68%

The *P*-values were used as a tool to check the significance of each of the coefficients for the model as well as each tested factor, which, in turn, are necessary to understand the pattern of the mutual interactions between the test variables. Interpretation of the data was based on the signs (positive or negative effect on the response) and statistical significance of coefficients at $P \leq 0.05$. Interactions between two factors could appear as an antagonistic effect (negative coefficient) or a synergistic effect (positive coefficient). It can be seen from the degree of significance that the linear effects, quadratic effects and interaction between X_1 (glucose) and X_3 (casein) are significant, meaning that they can act as limiting factors for protease production, and little variation in their values will alter the protease production rate. However, the ANOVA of the regression model demonstrates that the model is highly significant, as is evident from the very low *P*-value, which equals to zero.

Model adequacy checking and validation

Usually, it is necessary to check the fitted model to ensure that it provides an adequate approximation to the real system. Unless the model shows an adequate fit, proceeding with the investigation and optimization of the fitted response surface likely give poor or misleading results. Checking of the adequacy of the model needs all of the information on the lack of fit, which is contained in the residuals. Plotting these residuals against the fitted values of protease production (Figure 6) shows equal scatter of the residual data above and below the X-axis, indicating that the variance was independent of protease production, thus supporting the adequacy of the model fit.

In order to evaluate the relationship between dependent and independent variables and to determine the optimum levels of glucose and casein corresponding to the maximum protease production, a second-order polynomial model was proposed. The second-order polynomial equation that defines predicted protease response (*Y*) was obtained to be:

$$Y_{(\text{Protease activity})} = 8.05 + 1.911\ X_1 + 0.558\ X_3 + 1.987\ X_1^2 + 1.04\ X_3^2 + 1.432\ X_1X_3$$

To visualize the previous relationship between protease response and the interactions among the tested variables, graphical three-dimensional and Contour plots were generated (Figure 7) in order to determine the optimum conditions for protease production at the different levels of both glucose and casein. Exploring both figures reveal that the maximum protease production appeared at high levels of both X_1 and X_3 and low concentrations of both variables supported low protease production.

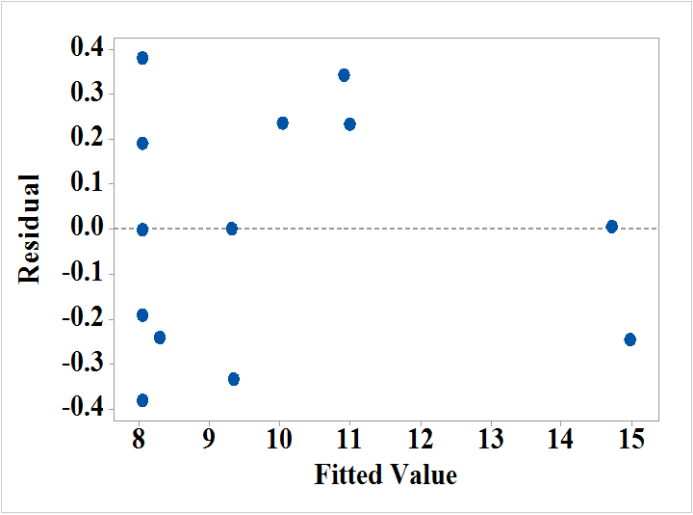

Figure 6 Plot of residuals against fitted values for protease production by *M. mucedo* strain SEE1.

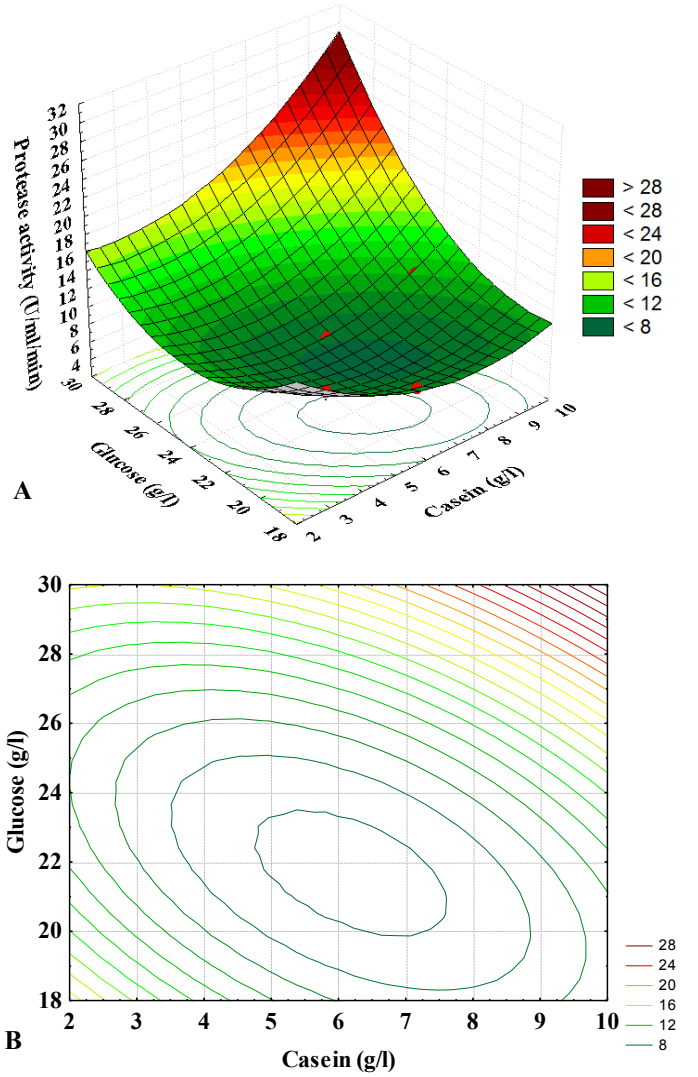

Figure 7 A) Three-dimensional response surface and B) Contour plot showing the interactive effects of independent variables (glucose and casein) on protease production by *M. mucedo* strain SEE1.

The previous model was experimentally validated, in order to determine the prediction accuracy of the model and to verify the optimization results, experiments were repeated in triplicates under optimized culture conditions obtained from fitted protease production model. Under these conditions, 14.90 ± 0.31 U/ml/min of protease was obtained. This value of protease activity corresponds very well to the values predicted by the fitted model 14.850 U/ml/min. Comparing protease production after CCD optimization, reveals 3.82 and 1.14-fold increment compared with before and after Plackett-Burman results, respectively.

Both glucose and casein proved to be the most potent nutritional factors affecting protease production. Glucose is simple carbon source, which is required, nearly in all growth stages of microorganism, the small amount encourage the growth of the fungus at the initial growth stage, so it could be considered as a limiting nutritional factor for both the microbial growth and protease production **(Rao *et al*., 2009; Abou-Ayana *et al*., 2015)**. However, other reports observed that the complex carbon source constitute better substrates for protease production than simple sugars such as glucose, which induced catabolic repression especially at the high concentrations **(Reddy *et al*., 2008)**. Glucose and casein were found to be the best carbon and nitrogen sources for protease production by *Mucor mucedo* DSM 809 **(Yegin *et al*., 2010)**.The presence of the substrate (casein) can induce protease secretion, however; high levels of the end products as an action of proteases, such as amino acids,NH^{4+}, and easily metabolizable sources of carbon may repress enzyme production **(Souza *et al*., 2015)**, therefore, protease productivity could be affected by the nature of carbon and nitrogen sources **(Vijayaraghavan *et al*., 2012)**. Finally, extracellular protease enzyme may be secreted constitutively at low levels regardless of the availability of a substrate **(Geisseler and Horwath, 2008)**.

Production of amino acids on the optimized fermentation medium

Nowadays, the increasing need to replace chemically synthesized compounds by environmental friendly ones using biological processes is driving the research for microbial factories. The industrial production of amino acids includes several examples of success stories using microorganisms to convert inexpensive substrates into valuable products. Herein, *M. mucedo* strain SEE1 was growing on optimized batch fermentation medium and the associated amino acids had been investigated. Obtained results indicated that 16 amino acids have been determined individually (Table 5).

Table 5 Amino acids profile produced by *M. mucedo* strain SEE1 on the optimized fermentation medium.

Amino acid	Amount (µg/ml) ± standard error
Alanine	138.7 ± 2.3
Arginine	580 ± 0.6
Aspartic acid	460.1 ± 2.9
Cysteine	36.0 ± 0.5
Glutamic acid	391.0 ± 1.7
Glycine	196.6 ± 1.2
Histidine	1155.3 ± 2.8
Isoleucine	580.0 ± 1.9
Leucine	386.5 ± 0.9
Lysine	897.4 ± 3.3
Phenylalanine	299.0 ± 0.7
Proline	114.0 ± 0.3
Serine	570.8 ± 1.0
Threonine	325.3 ± 3.0
Tyrosine	634.0 ± 2.6
Valine	490.8 ± 1.4

The content of histidine followed by lysine, tyrosine, arginine and isoleucine were significantly superior amino acids. However, alanine, cysteine, proline, glycine and phenylalanine showed less significant superiority, where, these amino acids are essential for growth of organisms, whereas, histidine, lysine, isoleucine and serine are non-essential. Contrarily, the amino acids, alanine and glycine were reported to be secreted by*Lactobacillus salivarius* **(Lee *et al*., 2014)**. Similar studies showed that the amino acids, L-phenylalanine, L-threonine and L-cysteine have been obtained by *E. coli* strains **(Ikeda, 2003)**, and L-glutamine wasproduced by *Corynebacterium glutamicum*, **(Kuethe *et al*., 2007)**. Other amino acids produced by *Corynebacteria* include L-valine, L-isoleucine, L-threonine, L-aspartic acid and L-alanine **(Eggeling and Sahm, 2011; Schneider *et al*., 2013)**.

Generally, microorganisms produce the 20 kinds of amino acids only in the amounts they need. They have a mechanism for regulating the quantities and qualities of enzymes to yield amino acids only in the amounts necessary for themselves **(Ivanov *et al*., 2013)**. Herein, *M. mucedo* strain SEE1 was directed, during the preceding optimization study, to accumulate several amino acids (up to 16) in its growth medium, encouraging the industrial production of such amino acids using this fungus and the proposed fermentation condition described earlier.

CONCLUSION

The fungus *Mucor* sp. was isolated according to its proteolytic activity on skim milk agar. Identification basis depend upon microscopic, morphological as well as 18S rRNA sequence confirmed this isolate namely, *M. mucedo* strain SEE1 under the accession number KP736529.Following the optimization procedure of protease, i.e. Plackett-Burman and central composite design, both glucose and casein have significant effect on the protease production. Amino acids produced (µg/ml) in the fermentation medium revealed in 16 amino acids. Histidine came in the first order (1155.3), followed by lysine (897.4), tyrosine (634), argentine (580) and isoleucine (580). Other amino acids e.g. alanine, cysteine and proline were measured in little amount. Ultimately, the fungus *M. mucedo* strain SEE1 can be exploited to produce some of amino acids in large scale, in which amino acids play important roles in the life, e.g. as animal feed additives, flavor enhancer, pharmaceuticals, cosmetics and production of lactic acid and some antibiotics.

CONFLICT OF INTERESTS

The author declares that there is no conflict of interests.

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EFFECT OF LACTIC ACID ON *Escherichia coli* O157:H7 AND ON COLOR STABILITY OF VACUUM-PACKAGED BEEF STEAKS UNDER HIGH STORAGE TEMPERATURE

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ABSTRACT

The effect of lactic acid (LA) addition on *Escherichia coli* O157:H7 survivability as well as the color stability was determined in vacuum-packaged beef steaks storage at 10°C for 50 days. *Longissimus dorsi* muscle was portioned into beef steaks and inoculated with *Escherichia coli* O157:H7. Afterwards, the samples were submitted at three treatments: without lactic acid addition or non-treated (NT); with 0.56 M (5%, v/v) (L5), and with 1.13 M (10%, v/v) (L10) of lactic acid addition. Same division was performed with samples non inoculated. All samples were package at vacuum and stored at 10ºC during 50 days. L10 demonstrated an efficient bacteriostatic effect ($P < 0.05$) against *E. coli* O157:H7 and total aerobic mesophilic bacteria. Nonetheless, LA induced a decrease ($P < 0.05$) in a^* values in samples after application and during storage, promoting discoloration of beef steaks. Therefore, L10 was efficient in controlling *E. coli* O157:H7 even at abusive storage temperatures. However, this decontamination treatment affects negatively the color stability of beef.

Keywords: Food preservation; Organic acid; *Longissimus dorsi*; Discoloration; EHEC

INTRODUCTION

Escherichia coli O157:H7 is an important foodborne pathogen, commonly harbored in cattle gut tract **(Bell, 2002; Caprioli *et al.*, 2005; Karmali *et al.*, 2010)** with high economic impact on industry and public health **(Scallan *et al.*, 2011)**. Thus, during cattle slaughter and carcass processing there is a considerable risk of fresh cut and processed product cross-contamination with *E. coli* O157:H7 **(Barkocy-Gallagher *et al.*, 2003; Huang and Sheen, 2011)** corroborating the link between outbreaks of this pathogen and beef **(Callaway *et al.*, 2009; Huang and Sheen, 2011)**.

In order to control microbial development, the meat industry usually uses organic acids on beef processing **(Buncic and Sofos, 2012)**. Lactic acid (LA) is a weak organic acid generally recognized as safe (GRAS) **(FDA, 2016)**, which is naturally present in the muscle tissue **(Siragusa, 1995; Muchenje *et al.*, 2009)** and is also produced by fermentative bacteria **(Martinez *et al.*, 2013; FDA, 2016)**. Acid decontamination with LA is considered a simple and valuable chemical strategy to improve food safety in meat products **(Ricke, 2003; Skandamis *et al.*, 2010)** due to the action immediately after application **(Anderson and Marshall, 2007)**, and also during storage, when the residual LA content exerts bacteriostatic effect on bacterial counts **(Dorsa *et al.*, 1998)**. However, despite LA bacteriological advantages, its application can promote surface color changes on meat **(Hunt *et al.*, 1999; Pipek *et al.*, 2005; Hosseini and Esfahani-Mehr, 2015)** resulting in economic losses **(Smith *et al.*, 2000)**.Beef color influences the consumer acceptability, which interfere on purchase decisions **(Suman *et al.*, 2010)**. This quality parameter is stated by myoglobin (Mb) redox state on meat: deoxymyoglobin (DMb; purplish-red color), oxymyoglobin (OMb; bright cherry-red color), and metamyoglobin (MMb; brown color). MMb results from the oxidation of myoglobin **(Suman *et al.*, 2007; Suman and Joseph, 2013)**, and the increase of MMb formation should be avoided in order to prevent the development of brown color on beef products surface. Therefore, it is important the use of package strategies, such as the vacuum package, in order to maintain meat color **(Kerry *et al.*, 2006; Zhou *et al.*, 2010)**, wholesome and safe **(Leistner, 2004; John *et al.*, 2005; Han, 2014)**, during storage.

Previous studies **(Youssef *et al.*, 2012; Harris *et al.*, 2012; Youssef *et al.*, 2013; Li *et al.*, 2015; Blagojevic *et al.*, 2015)** examined the effect of lactic acid concentrations up to 5% combined with temperature to evaluate the reduction of *E.coli* O157:H7 during storage, however, there is a little information about the inhibition effects of use lactic acid concentrations higher than 5% on vacuum package beef steaks, during prolonged storage at 10ºC.Therefore, the goals of the present research were 1) to evaluate the most efficient lactic acid concentration (0.56 M; 5%, v/v or 1.13 M;10%, v/v) for *E. coli* O157:H7 reduction and 2) the effect of lactic acid application on pH and surface color stability of vacuum packaged beef steaks (*Longissimus dorsi*) during 50 days of storage at 10ºC.

MATERIALS AND METHODS

Experimental design

Longissimus dorsi (LD) beef (21kg) was obtained between the 6th thoracic rib and the 6th lumbar vertebrae from a commercial processing facility inspected by the federal government (Colatina, Espírito Santo Brazil) after 36 h post-mortem. LD was transported under refrigeration (0°C) to the Meat Laboratory of Universidade Federal Fluminense where was portioned into beef steaks (100 g each; area: 256 cm²; 9.5 cm diameter and 2.0 cm thick approximately). Samples were equally divided in two groups (non-inoculated and inoculated). Both groups were randomly subjected to surface application of LA solution at either 0.56 M (5%, v/v) (L5) or 1.13 M (10%, v/v) (L10) or were not treated (NT). After LA treatment, samples were vacuum packed and stored for 50 days at 10°C. The abusive temperature model used to stimulate *E. coli* O157:H7 growth was performed follow the indication of **Hwang *et al.* (2014)**. Three trials (n = 3; 7 kg each trial) were carried out, totaling 70 unit samples (100g) per trial. Physicochemical and bacteriological analyses were performed at days 0, 3, 15, 30, and 50 of storage.

Raw beef inoculation

Escherichia coli O157:H7 strain (EDL 933) **(Gobert *et al.*, 2007)** was provided by the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil). The lyophilized

inoculum was added to brain heart infusion broth (BHI) (Difco®, Detroit, MI) and activated at 37°C for 24 h. Then, 1 ml aliquots were transferred to test tubes containing 9 ml of BHI, and incubated at 37ºC for 18 h **(Meng *et al*., 2001)**. The inoculum was calculated using an UV spectrophotometer (Smartspec Plus, BioRad, Hercules, CA) at 600 nm and a concentration of 10^8 cells/ml was detected **(Lázaro *et al*., 2014)**. The inoculum concentration was confirmed by spread plating on Plate Count Agar (PCA) (Merck®, Darmstadt, Germany) and O157:H7 Agar (Merck®, Darmstadt, Germany) **(Alegre *et al*., 2010)** achieving 6 log CFU/g on meat.

The inoculation was performed on beef steaks (100g), aseptically transferred to plastic packages BBL4 (Cryovac®, Saddle Brook, NJ). In this conditions 0.5 ml of *Escherichia coli* O157:H7 inoculum were pipetted on both sides of the beef surfaces (1ml per sample) **(Alegre *et al*., 2010)**. The inoculated samples were massaged for 1 min and remained 30 min at 22 ºC to allow the inoculum attachment to the meat surface **(Huang and Chen, 2011; Mahmoud, 2014)**. After inoculation, the samples were treated with different solutions of lactic acid.

Lactic acid treatment

Solutions of 0.56 M (5%, v/v) (L5) and 1.13 M (10%, v/v) (L10) of lactic acid were prepared from 85% lactic acid (Fisher Scientific, Pittsburgh, PA) using sterile deionized water. Non-inoculated and inoculated LD steaks were pipetted on each side with 2.5 ml of L5 or L10 solutions at room temperature, vacuum sealed, and stored at 10ºC.

Bacteriological evaluation of inoculated beef steaks

The inoculated steaks (25g) were aseptically transferred to sterile bags containing 225 ml of peptone saline (0.10% peptone in 0.85% NaCl) and homogenized utilizing a stomacher (Stomacher 80, Seward Ltd., London, United Kingdom) for 2 min. The homogenized samples were serially diluted in peptone saline and plated onto Petri dishes containing plate count agar (PCA) to evaluate total aerobic mesophilic bacteria (TAMB) **(APHA, 2001)**, and O157:H7 Agar (Merck® KGaA, Darmstadt, Germany) to determine *Escherichia coli* O157:H7 counts **(Alexa *et al*., 2011)**. Incubation was performed at 37°C for 24 h and results were expressed as log CFU/g.

Physicochemical evaluation of non-inoculated beef steaks

Non-inoculated steaks (NT, L5 and L10) were used to evaluate pH values and instrumental color parameters. The analysis of pH was performed using a Handylab 1 (SchottGlaswerke, Mainz, Germany) pH meter previously calibrated with buffer solutions at pH 4.0 and 7.0 **(AOAC, 2012)**.

Instrumental color parameters were evaluated using a Minolta CM-600d spectrophotometer (Konica Minolta Sensing Inc., Osaka, Japan) with 8 mm diameter measuring aperture, illuminant A, and 10° standard observer **(Canto *et al*., 2015)**. Meat color was measured at the surface of non-inoculated steaks (NT, L5, and L10) after blooming for 10 min at 22°C. The parameters determined were: lightness (L^* value), redness (a^* value), yellowness (b^* value), and ratio of reflectance 630 to 580 nm (R630/580) **(AMSA, 2012)**.

Statistical analysis

A one-way analysis of variance was performed to evaluate the effect of LA application, LA concentration and days of storage on LD steaks on bacteriological (inoculated) and physicochemical (non-inoculated) parameters, using XLSTAT statistical software (Version 2014.5.03, Addinsoft, Inc., Brooklyn, NY, USA). Differences among means were tested using Tukey's test with 95% of confidence level.

RESULTS

Regarding the bacteriological evaluation, the inoculated LD steaks treated with LA solutions (L5 and L10) did not exhibit difference ($P > 0.05$) on *Escherichia coli* O157:H7 and total aerobic mesophilic bacteria (TAMB) counts when compared to non-treated (NT) samples on day 0 of storage (Table 1). However, from day 3 until day 50 of storage, group L10 showed a significant reduction ($P < 0.05$) of *E. coli* O157:H7 and TAMB counts compared to NT, suggesting the bacteriostatic effect of LA, even under abusive temperature.

Table 1 *Escherichia coli* O157:H7 and total aerobic mesophilic bacteria (TAMB) counts (log cfu/g) on *Longissimus dorsi* steaks treated with lactic acid storage at 10 ºC during 50 days.

Microorganism		Days of storage					
	Treatment *	0	3	15	30	50	*SEM*
Escherichia coli O157:H7	NT	6.0 ^{aB}	6.6 ^{aAB}	6.8 ^{aAB}	7.2 ^{aA}	7.7 ^{aA}	*0.07*
	L5	5.2 ^{aB}	5.6 ^{abAB}	5.7 ^{abAB}	6.2 ^{bAB}	6.6 ^{bA}	*0.07*
	L10	5.0 ^{aAB}	5.2 ^{bA}	4.9 ^{bAB}	4.9 ^{cAB}	4.1 ^{cB}	*0.07*
	SEM	*0.08*	*0.09*	*0.11*	*0.05*	*0.05*	
TAMB	NT	6.8 ^{aD}	7.4 ^{aCD}	8.4 ^{aBC}	9.5 ^{aAB}	10.4 ^{aA}	*0.16*
	L5	7.0 ^{aC}	7.5 ^{aABC}	7.2 ^{bBC}	8.7 ^{abA}	8.6 ^{bAB}	*0.14*
	L10	6.1 ^{aBC}	6.1 ^{bBC}	5.9 ^{cC}	7.2 ^{bAB}	6.9 ^{cABC}	*0.09*
	SEM	*0.20*	*0.01*	*0.08*	*0.19*	*0.17*	

Legend: $^{a—c}$ Means in a column without common superscripts are different ($P < 0.05$).
$^{A—D}$ Means in a row without common superscripts are different ($P < 0.05$).
* NT= Non-treated samples, L5= samples treated with 0.56 M (5%, v/v) LA solution, and L10= samples treated with 1.13 M (10%, v/v) LA solution.
SEM= standard error of the mean.

Lactic acid treatment decreased ($P < 0.05$) pH values (Table 2). In terms of LA concentration on days 0 and 15, NT exhibited the greatest ($P < 0.05$) pH values, followed by L5, and L10 which presented the lowest ($P < 0.05$) ones. On the other hand, on days 30 and 50 no difference ($P > 0.05$) was observed between pH values of L5 and L10. In addition, NT, L5, and L10 exhibited an increase ($P < 0.05$) pattern of pH, during the storage period.

Table 2 shows the instrumental color parameters. LA did not exhibit an immediate effect ($P > 0.05$) on L^* values. From day 15 to 50, L10 decreased ($P < 0.05$) sample lightness while L5 remained similar ($P > 0.05$) to NT samples. In addition, during storage L5 and L10 exhibited L^* value decrease ($P < 0.05$) whereas, NT samples did not exhibit ($P > 0.05$) variation. Regarding meat redness (Table 2), LA concentration affected ($P < 0.05$) a^* values. NT demonstrated the greatest ($P < 0.05$) a^* values, L5 intermediate, and L10 the lowest ones ($P < 0.05$) indicating a concentration-dependent pattern. From day 0 to 50, a^* values of L5 and L10 LD steaks decreased ($P < 0.05$), although NT samples did not demonstrated ($P > 0.05$) variation on redness during storage. In terms of yellowness (Table 2), LA treatment promoted an increase ($P < 0.05$) of b^* values after application, whereas on days 15, 30 and 50, L10 exhibited the lowest ($P < 0.05$) b^* values and no difference ($P > 0.05$) was observed on NT and L5 samples.

Regarding LD steak surface color stability (Table 2), LA concentration and storage period affected ($P < 0.05$) R630/580 values. During all storage days, NT exhibited the greatest ($P < 0.05$) R630/580 values. At days 0 and 15 of storage, L10 demonstrated the lowest ($P < 0.05$) R630/580 values, whereas at days 30 and 50 similar results were obtained for L5 and L10 ($P > 0.05$). In addition, NT, L5, and L10 demonstrated decrease ($P < 0.05$) of R630/580 values during storage.

Table 2 Means of pH and instrumental color parameters values on *Longissimus dorsi* steaks treated with lactic acid storage at 10 ºC during 50 days.

Treatment *	Days of storage				
	0	15	30	50	*SEM*
pH					
NT	5.38 ^{aBC}	5.33 ^{aC}	5.50 ^{aAB}	5.63 ^{aA}	*0.00*
L5	4.46 ^{bC}	4.84 ^{bB}	5.26 ^{bA}	5.37 ^{bA}	*0.01*
L10	4.05 ^{cC}	4.66 ^{cB}	5.19 ^{bA}	5.21 ^{bA}	*0.01*
SEM	*0.01*	*0.00*	*0.01*	*0.01*	
L* (lightness)					
NT	38.34 ^{aA}	37.76 ^{aA}	38.25 ^{aA}	37.83 ^{aA}	*1.89*
L5	40.97 ^{aA}	37.12 ^{aB}	36.83 ^{aB}	35.01 ^{aB}	*1.14*
L10	38.73 ^{aA}	29.72 ^{bB}	30.19 ^{bB}	30.00 ^{bB}	*1.04*
SEM	*1.60*	*0.84*	*1.39*	*1.60*	
a* (redness)					
NT	22.09 ^{aA}	21.32 ^{aA}	21.22 ^{aA}	21.85 ^{aA}	*0.16*
L5	20.77 ^{bA}	14.19 ^{bB}	13.27 ^{bB}	13.17 ^{bB}	*0.44*
L10	16.10 ^{cA}	11.43 ^{cB}	11.06 ^{cB}	9.18 ^{cC}	*0.40*
SEM	*0.18*	*0.28*	*0.34*	*0.54*	
b* (yellowness)					
NT	14.83 ^{bA}	13.73 ^{aAB}	13.67 ^{aB}	13.83 ^{aAB}	*0.18*
L5	15.79 ^{aA}	14.22 ^{aB}	13.21 ^{aB}	12.94 ^{aB}	*0.27*
L10	15.81 ^{aA}	9.76 ^{bB}	9.81 ^{bB}	7.84 ^{bC}	*0.47*
SEM	*0.12*	*0.13*	*0.49*	*0.49*	
R630/580 (ratio of reflectance)					
NT	4.48 ^{aA}	4.22 ^{aAB}	4.18 ^{aAB}	4.08 ^{aB}	*0.02*
L5	3.22 ^{bA}	1.76 ^{bB}	1.53 ^{bB}	1.53 ^{bB}	*0.04*
L10	1.82 ^{cA}	1.47 ^{cB}	1.41 ^{bBC}	1.34 ^{bC}	*0.00*
SEM	*0.05*	*0.00*	*0.00*	*0.02*	

Legend: $^{a—c}$ Means in a column without common superscripts within a attribute are different ($P < 0.05$).
$^{A—C}$ Means in a row without common superscripts are different ($P < 0.05$).
* NT= Non-treated samples, L5= samples treated with 0.56 M (5%, v/v) LA solution, and L10= samples treated with 1.13 M (10%, v/v) LA solution.
SEM= standard error of the mean.

DISCUSSION

The reduction of bacterial count in samples treated with LA solution is probably due to lactate anion accumulation **(Van Immerseel *et al*., 2006)** which promoting membrane damage, ATP depletion, and disrupting nutrient transport **(Cherrington *et al*., 1991)**. In addition, weak organic acids such as LA are considered lipophilic and capable of passing through cellular membrane, acidifying the bacterial cytoplasm, affecting homeostasis and influencing *E. coli* O157:H7 and TAMB growth **(John *et al*., 2005)**.

In agreement with present results, **Shrestha and Min (2006)** reported a concentration-dependent pattern on TAMB reduction from the 4th day of storage on fresh pork ham treated with solutions of LA at 1%, up to 6% (v/v). In partial agreement with present results, **Zeitoun and Debevere (1992)** observed that TAMB and *Enterobacteriaceae* counts remained constant in fresh chicken legs treated with lactic acid at 5% (v/v) and 10% (v/v) during at least 14 days of storage. On the other hand, **Harris *et al.* (2012)** observed a reduction of 0.8 log CFU/g on *E. coli* O157:H7 and TAMB counts in ground beef treated with lactic acid at 5% (v/v) on day 0. In addition, **Pittman *et al.* (2012)** reported *E. coli* O157:H7 reduction of 1.6 log CFU/g on beef carcasses after 24 h of decontamination using 5% (v/v) LA. Furthermore, **Mahmoud (2014)** observed reductions of 2.8 and 3.4 log CFU/g of gram negative bacteria in inoculated oysters treated with LA of 0.5 M and 1.1 M immediately after LA application, contrasting with our results. These contrasts could be attributed to differences on LA application methods and type of matrix.

In relation to pH values, the LA decreased them immediately after application, probably due to a proton imbalance in meat muscle caused by LA solutions **(Goli *et al*., 2011)**. Our results are in agreement with **Naveena *et al.* (2006)**, which observed a decrease in pH values in buffalo meat treated with 2% (v/v) of lactic acid, and with **Shrestha and Min (2006) and Grajales-Lagunes *et al.* (2012)** which treated pork meat with LA solutions ranging from 1% to 6% (v/v) at the beginning of the storage period. Moreover, the increase pattern in pH values observed during storage can be attributed to amino acids decarboxylation in response to an acid stress **(Halász *et al*., 1994).** The decarboxylation is a cell mechanism to maintain the homeostasis, and the loss of a carboxylic group results in the formation of basic molecules, such as amines, that increases the pH of samples **(Pereira *et al*., 2009)**.

Regarding surface color stability, *L** values decrease during storage on L5 and L10 can be attribute to denaturation of myofibrillar proteins promoted by LA application affecting the water-holding capacity. The water dispersed among the muscle fibers may influence meat surface reflectance **(Aktaş and Kaya, 2001)**. A decrease in *L** values was also observed in fresh pork ham treated with solutions of LA during storage **(Shrestha and Min, 2006)**.

Lactic acid affected *a** values probably due to heme pigment oxidation induced by pH shift **(Hunt *et al*., 1999; Pipek *et al*., 2005)**. The change on pH leads to premature browning of beef as a consequence of MMb accumulation **(Smulders and Greer, 1998)**. In addition, the decrease on *a** values during storage can be explained by myoglobin oxidation, promoting a decrease in redness **(Carlez *et al*., 1995)**. Meat redness decrease was reported in *Longissimus dorsi* beef **(Aktaş, and Kaya, 2001)**, beef carcasses, **(Pipek *et al*., 2005)**, beef trimmings **(Harris *et al*., 2012)** and pork ham **(Shrestha and Min, 2006)** treated with 1.5, 2.0, 5.0 and 6.0% (v/v) of LA solutions during storage, respectively.

The acid treatment can also affect the perception of *b** values **(Friedrich *et al*., 2008)**. LA application increase protein denaturation and exudate release, as function of pH drop, which potentially explains the variations observed on meat yellowness **(Greer and Dilts, 1995)**. The decrease in *b** values in beef carcasses treated with 2% (v/v) LA solutions during storage was also previously observed **(Pipek *et al*., 2005; Mohan *et al*., 2011)**.

Color stability can be estimated based on the ratio of reflectance at 630 nm to 580 nm (R630/580). High ratio values indicate greater redness reflected by the greater OMb than MMb content, and a ratio value of 1.0 represents a meat surface with essentially 100% of MMb **(Strange *et al*., 1974)**. In agreement with the present results, previous researches **(Stivarius *et al*., 2002; Pipek *et al*., 2005; Mohan *et al*., 2011)** observed a decrease in R630/580 values in beef treated with 2% (v/v), 5% (v/v) of LA, as well as organic acid solutions, respectively. In addition, a decrease in R630/580 values on *Longissimus lumborum* and *Psoas major* muscles was also observed due to storage period **(Joseph *et al*., 2012)**.

CONCLUSION

LA solution at 1.13 M (10%, v/v) promotes an efficient control on inoculated *E. coli* O157:H7 vacuum packaged steaks during storage at 10ºC. In spite of the technological potential of LA decontamination for meat industry, this method promoted meat discoloration with a premature browning on *Longissimus* beef surface.

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POLYPHASIC ANALYSIS OF TWO THERMOTOLERANT, AND EXOZYMES PRODUCING *GEOBACILLUS* SPECIES FROM HOT SPRING OF NEPAL

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ABSTRACT

Background and Aim: Nepal's geothermal areas are considered as uncharted place for microbiological and biotechnological researchers since not many researches has been conducted on these areas. The main aim of this study was to isolate thermophilic bacteria from hostspring of Nepal. **Methods:** From one of the hottest natural thermal spring of Nepal, Bhurung Tatopani, 20 different bacteria were isolated and Characterized by both culture dependent and independent approaches. **Results:** Morphological and biochemical tests along with polyphasic analysis of these thermal isolates confirmed that two of these species belongs to *Geobacillus* sp. The isolates THG1 and THG2 were found to be gram variable and rod shaped with smooth colony. They were found to be osmotolerant up to 2% NaCl and thermotolerant with optimum temperature of 72°C. They showed significant production of various industrially important enzymes like amylase, lipase, protease and cellulase. 16S rRNA amplification was also performed and sequenced which revealed that the isolate THG1 has 99% similarity with *Geobacillus kaustophilus* and THG2 has 99% similarity with *Geobacillus thermoleovorans*. THG1 and THG2 16S rRNA sequences are deposited in genebank with accession id KP764939 and KP764940. For the further confirmation and deeper analysis, fatty acid analysis (FAME) was also performed and their fatty acid composition profile was also created. **Conclusion:** From the Bhurung hotspring two unique *Geobacillus* species were isolated.

Keywords: Bhurung Tatopani, Geobacillus, FAME, Thermotolerant

INTRODUCTION

Hot springs are unique hydrothermal features formed when underground water, heated due to geothermal energy. Generally hot springs discharge water which is heated by magma close to the earth's surface in volcanic areas. Some, however, are not related to volcanic activity. In such cases, the heating of water results from convective circulation and thermal springs from Nepal are examples of this type of phenomenon. More than twenty-eight geothermal manifestations occurs in Nepal, stretching right across a southeast-northwest elongated region. Some of the geochemical studies on these thermal springs have been conducted in the 80's (**Bhattarai and Bashyal, 1983; 3. Bhattarai,1980; 4. Bashysal, 1984**). Geothermometer temperatures, ionic balance and discharge enthalpy has been calculated from the chemical data using the Program called WATCH (**Arnorsson *et al.*, 1982; Bjamason, 1994**). Very few microbial and Biotechnological researches have been conducted in the Hot springs locations of Nepal. Since thermal springs are rich with numerous thermophiles, exploration of these microrganisms is needed for revealing the potentials of different thermophiles to be used through Biotechnology. Very few researches was conducted by NAST but not much of information was available in literature regarding presence of any noble and industrially potential thermophiles in various hotsprings. **Adhikari *et al.* (2016)** reported 15 different bacillus species from Bhurung tatopani with high extracellular enzyme production capacity and potential for industrial upgrades.
Apart from this, different exploration on hot springs located at different places of world has been conducted for isolating different important microorganisms. Many thermophilic bacterial strains were isolated from hot springs but the most common and significant finding in the field of thermophilic bacteria belongs to the genus *Thermus* (**Brock and Freeze, 1969; Kristjansson and Alfredsson, 1983; Kristjansson *et al.*, 1986; Kanasawud *et al.*, 1992**) *Bacillus* (**Marsh and Larsen, 1952**) and **Nazina *et al.*, (2001)** rearranged and grouped Gram positive, rod-shaped, endospore-forming thermophilic bacilli into the genus Geobacillus.
Geobacillus are unique bacterial species that are rod-shaped, occurring either singly or in short chains and motile by means of peritrichous flagella (**Nazina *et al.*, 2001**). Their cell wall structure is Gram-positive, but the Gram-stain reaction may vary between positive and negative. They can also be aerobic and sometimes facultative anaerobic. The growth temperature ranges from 37°C to 75°C with an optimum at 55°C to 65°C. They have ability to grow at wide pH range of 6.0 to 8.5 with an optimum growth at pH 6.2 to 7.5. Industrial interests in Geobacillus species has arisen from their potential applications in biotechnological processes, for example as sources of various thermostable enzymes, such as proteases (**Sookkheo *et al.*, 2000**), amylases (**Rao and Satyanarayana, 2003**), lipases (**Lee *et al.*, 2001**), pullanases (**Messaoud *et al.*, 2002**) and xylanase (**Sharma *et al.*, 2007**). Geobacillus species are widely distributed and readily isolated from different habitats (**Nazina *et al.*, 2001**), with a rapid increasing industrial interest towards their thermostable gene products (**Schallmey *et al.*, 2004**). Therefore, molecular characterization and study of its diversity with phylogenetic relations is not only regarded as a taxonomical concern, but is also a necessity for exploiting its biotechnological potential as a whole.
Nepal has a unique location which is diverse in extreme environments including the Highest mountain in the world, the lowest place on earth, in addition to several hot springs. Therefore, the aim of the current study was to isolate, identify and characterize thermophilic Geobacillus bacteria from 2 naturally occuring thermal springs in Bhurung, Nepal using phenotypic (morphological, physiological and biochemical) and genotypic methods (16S rRNA gene sequencing). Moreover, the extracellular thermostable enzymes of the obtained isolates were also identified.

MATERIAL AND METHODS

Study site

Bhurung Tatopani is located on Dhaulagiri Zone, Myagdi District, Singa V.D.C. Ward No 4. It is one of the hottest amongst 20 hot springs in Nepal and was a virgin area for microbial exploration. Heavy smell of sulphur gas and stale egg was present around the sources and upon close observation, the reservoir exhibited a green color. The study site was located at Latitude: 28°29'25.3" and Longitude: 83°37'54.06" with estimated terrain elevation 2262 meters above sea level. Though there are 3 different sources of hot springs, we chose two springs study site due to their high temperature and remote location.

Sample collection

Insitu measurement of temperature and pH was performed within the sampling site. The water samples were collected in sterile glass vials and labeled individually. The sediments with the soil and microbial community were also collected in glass vials and kept into thermos without fluctuating temperature. All the vials were brought into SANN laboratory for further analysis.

Enrichment and isolation

Castenholz D basal salts medium supplemented with tryptone and yeast (TYE) was used as culture media with slight modifications. Water samples was inoculated into liquid medium and incubated at 72°C for 2–3 days in a shaker incubator. Turbid cultures were spread on the same medium solidified with phytagel (1–1.5%) and incubated at the same temperature until yellow or non pigmented colonies appear and can be isolated. After 2-3 days, yellow and colorless colonies easily observed were picked for further purification and analysis.

Identification and characterization of the isolate

The isolated strain's cell morphology and motility was examined by optical microscopy (BX40; Olympus) and growth characteristics were also studied on soild media. Based on Gram's staining the isolates were found to be Gram-variable and microscopic observation revealed rod shaped which are arranged in chain. Different biochemical tests like motility determination, endospore formation, catalase, IMViC, and oxidase tests were also performed. Morphological observation and biochemical tests indicated that the isolated bacteria belonged to be Geobacillus sp. Similarly different physiological characterization was also performed for testing degree of osmotolerant, pH tolerant and thermotolerant. Determination of optimum growth by turbidity measurements was also performed.

Extracellular enzyme production

Bacterial isolates were screened for their ability to produce different thermostable extracellular enzymes like amylases, lipases and cellulases. The culture extracts grown in different single carbon source media at 70°C as well as freshly grown cultured of isolates was used for enzyme screening. Tween 80 NA medium was used to study lipase activity. Amylase activity was determined using starch agar plates and positive results was confirmed by incubating the plates and exposing to iodine solution. Similarly, Cellulase activity was studied on carboxy methyl cellulose (CMC 1%) agar.

Molecular identification

Genomic DNA extraction was peformed using CTAB manual method as well as using promega DNA isolation kit and purified using protocol from Sambrook and Russell (**Sambrook and Russell, 2001**). In order to access its purity, UV spectrophotometry was performed at A260/A280 ratio. The extracted DNA was then used as template for amplifying the gene encoding for the 16S rDNA (**Edwards *et al.*, 1989**) using bacterial universal primer.

Table 1 Universal primer for 16S rRNA amplification

Primer	Sequence (5'-3')	Primer length	GC%	Sequence amplified
518F	CCAGCAGCCGCGGTAATACG	20	65	1500bp
800R	TACCAGGGTATCTAATCC	18	44.44	1500bp

The reaction volume of 25µl containing 2ul of 20ng/ul template DNA, 1unit of Tag DNA polymerase , 75 mM of MgCl2 , 200 µM of DNTPs, 2.5 µl of 10X PCR buffer and 5 pmol of each Primer. The Amplification protocol was programmed with 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension of 7 min. Gel electrophoresis was performed using 2% agarose gel and the amplified amplicon was observed under the gel documentation system. The PCR products were purified using the Promega PCR purification Kit protocol according and sequenced in Macrogen Inc. Korea.

Phylogenetic analysis

The sequenced 16S rRNA sequences obtained from Macrogen were first manually edited using Sequencher Software 5.3 in order to remove ambiguity in the sequences. Since bidirectional sequencing was carried out, single contigs was built using the same software and exported as text format. The edited sequences were then compared with the nucleotide database using Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information (NCBI). The closest relatives (high similarity) with the query sequences were retrieved and aligned using MUSCLE. Aligned sequences were analysed and phylogenetic tree was constructed using Mega 6.0 (**Tamura *et al.*, 2013**).
Neighbor-Joining method and maximum likelihood method (**Saitou and Nei, 1987; Tamura *et al.*, 2013**) was used to show the evolutionary relationships of these taxa, The optimal tree with the sum of branch length = 0.81476834 was drawn and the percentage of replicate trees in
which the associated taxa clustered together in the bootstrap test (1000 replicates) shown next to the branches. Bootstrap analysis using Mega 6, was performed to attach confidence estimates for the tree topologies (**Felsenstein, 1985**). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Maximum Composite Likelihood method was used to compute the evolutionary distances.

Fame analysis

Bacterial isolates were also identified by using Sherlock Microbial Identification System (MIS), MIDI in Royal Life Science Pvt Ltd, India. This is a unique system that identifies microorganisms based on their fatty acid methyl ester (FAME) patterns. FAME profiles are then compared to a standard database and most likely hits are then presented to the user. The database includes aerobic bacteria, anaerobic bacteria and yeasts. These FAME's were analyzed using Gas Chromatography with the help of MIDI Sherlock software for FAME. Aerobic library (RTBSA 6.0) was referred for the analysis.

RESULTS

Physical characteristics of sampling sites

During sampling time there was a heavy smell of sulphur gas around the sources and the reservoir exhibited a green colour. The physical parameters at the source of thermal spring during the sampling of the thermal springs are mentioned in (Table 2). These values are the raw figures of the conditions at the sampling sites of the thermal waters at Bhurung tatopani during the sampling time.

Table 2 Physical parameters at the hot springs of Bhurung tatopani during the sampling time

Sampling /Conditions parameters	Source 1	Source 2
Atmospheric temperature (ºC)	18	18
Hot spring water temperature (ºC)	58	70
Stream waterway temperature (ºC)	55	65
Surface elevation	2262 meters	2262 meters
pH	7.5	8.2
Conductivity	1	1
Position	28°29'25.3"N/ 83°37'54.06"E	28°29'25.3"N/ 83°37'54.06"E

Morphological characterization of isolates

Table 3 Morphological characterization of isolates

Strains	Colony colour	Cell shape	Gram reaction
THG-1	Yellow	rod	variable
THG-2	Yellow	rod	variable

Physiological characterization

Growth at various concentration of sodium chloride

Table 4 Growth of thermophilic isolates from the hot springs of Bhurung at varied NaCl concentration.

Strains	0% Nacl	1% Nacl	2% Nacl	3% Nacl	4% Nacl	5% Nacl
THG1	+++	+++	++	-	-	-
THG2	+++	+++	+	-	-	-

Legend: +++ High growth , ++ Moderate growth, - Negative growth

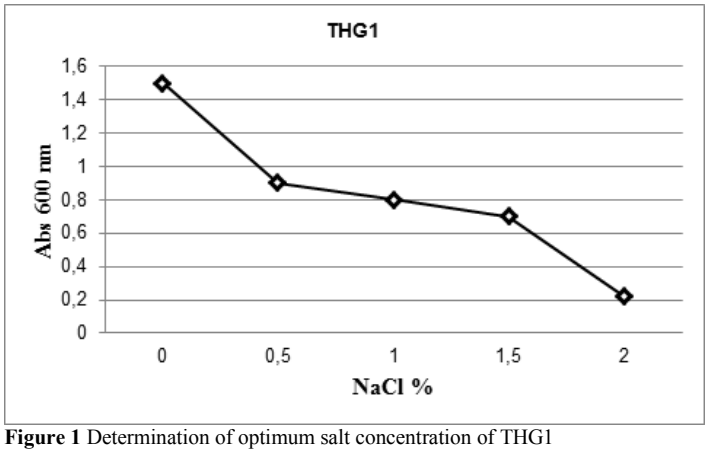

Figure 1 Determination of optimum salt concentration of THG1

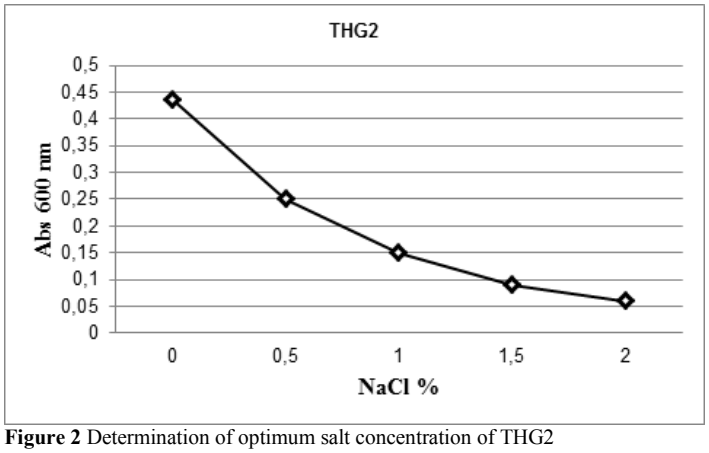

Figure 2 Determination of optimum salt concentration of THG2

Growth of isolates at different temperature

Table 5 Growth of thermophilic isolates from the hot springs of Bhurung at varied temperature.

Temp / Strains	35ºC	45ºC	55ºC	65ºC	70ºC	75ºC	80ºC
THG1	-	+	+	+	+++	+	-
THG2	-	+	+	+	+++	+	-

Legend: +++ High growth , + Moderate growth, - Negative growth

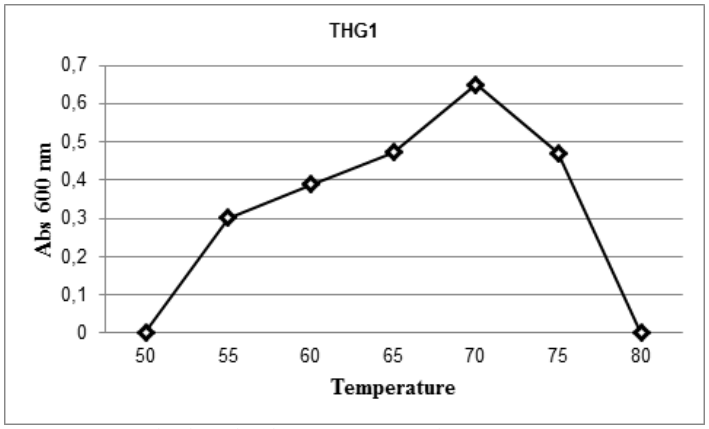

Figure 3 Determination of optimum temperature for THG1

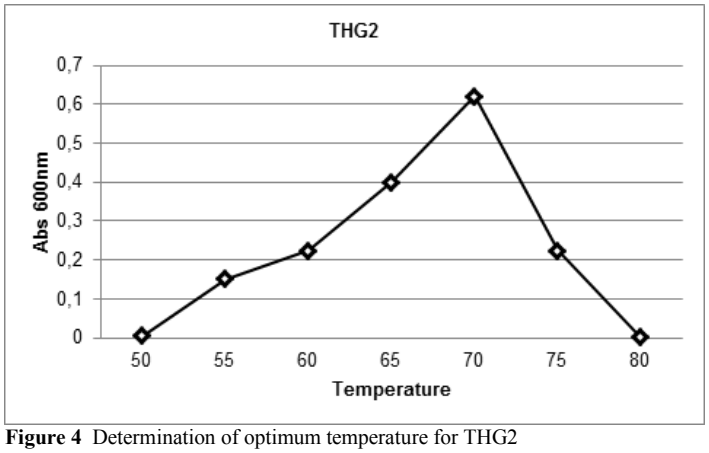

Figure 4 Determination of optimum temperature for THG2

EFFECT OF ph ON THE ISOLATES

Table 6 Growth of thermophilic isolates from the hot springs of Bhurung Tatopani at varied pH.

pH / Strains	5	6	7	8	9
THG1	-	-	++	+++	-
THG2	-	-	++	+++	-

Legend: +++ High growth , + Moderate growth, - Negative growth

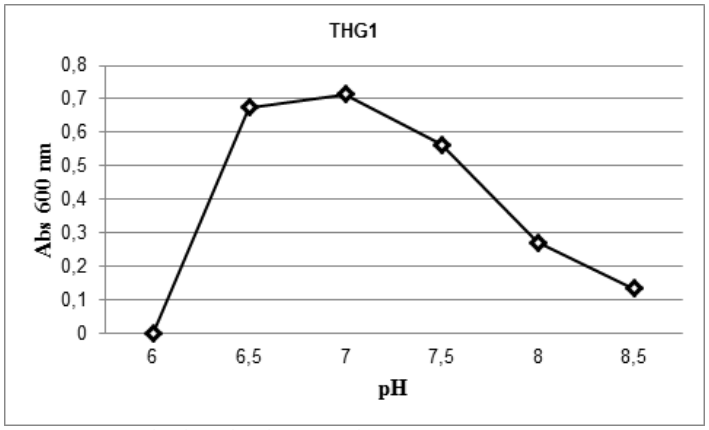

Figure 5 Determination of optimum pH for THG1

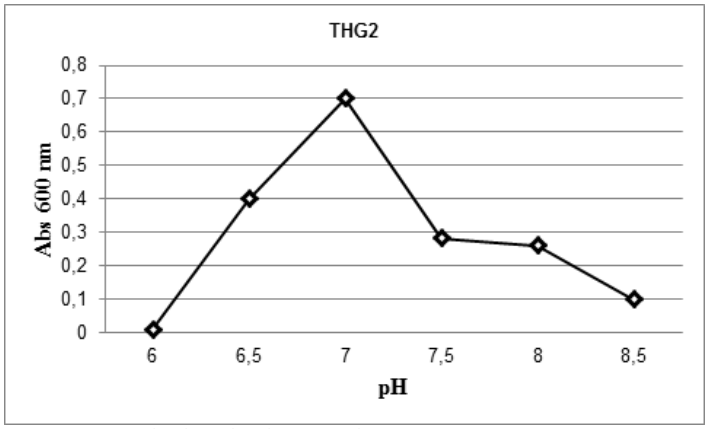

Figure 6 Determination of optimum pH for THG2

Determination of optimum growth of two strains by turbidity measurements

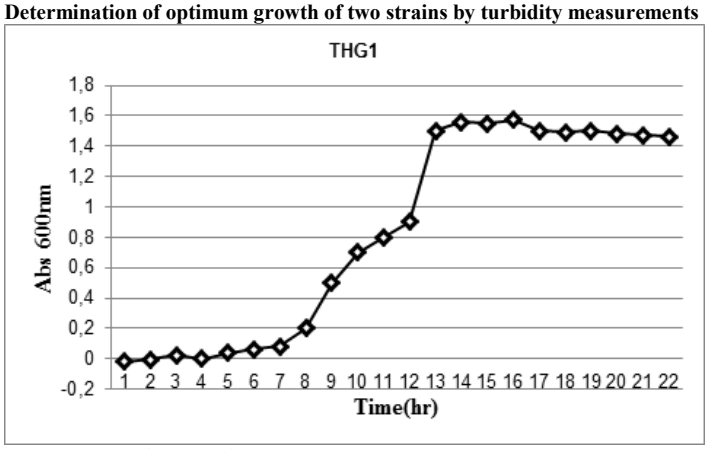

Figure 7 Growth curve of THG1

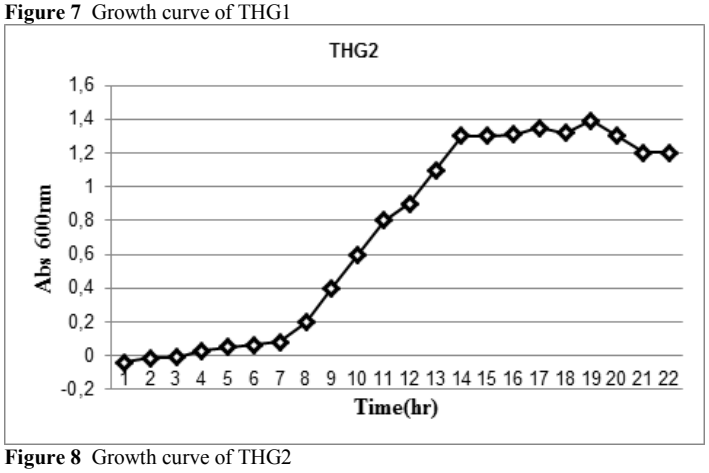

Figure 8 Growth curve of THG2

Biochemical characterization

Table 7 Biochemical characteristics of strains THG1 and THG2

Characteristics	THG1	THG2
Pigmentation	Yellow	Yellow
Colonies	Compact	Compact
Optimum Temp°C	70	70
Growth at/on:		
Growth in 1% Nacl	+	+
Growth in 3% Nacl	-	-
Presence of:		
Oxidase	+	+
Catalase	-/+	+
ß-Galactosidase	+	+
Urease	-	-
IMVIC		
Indole	-	-
MR/VP	-	-
Citrate	+	+
Hydrolysis of :		
Starch	+	+
Gelatin	+	+
Casein	+	+
Tween 20	+	+
Utilization of:		
D-Glucose	+	+
D-Fructose	+	+
D-Galactose	+	+
D-Xylose	+	+
D-Mannose	nd	nd
Cellulose	+	+
Lactose	+	+
Sucrose	-	-
Raffinose	-	-
Citrate	+	+
Arginine	+	+
Proline	+	+
Ornithine	+	+
L-Serine	+	+
Glycerol	+	+
Sensitive to:		
Amoxicillin(10µg)	+	+
Ampicillin(10µg)	+	+
Neomycin(30µg)	+	+
Oxacillin(10µg)	-	-
Penicillin(10IU)	+	+
Streptomycin(10µg)	+	+
Vancomycin(30µg)	+	+
DNA G+C content (mol%)	59.77	59.022

Legend: + Positive result; - Negative result or No growth; nd-not determined.

Enzyme production

Table 8 Detection of extracellular enzymes for the 2 isolates at 70°C.

Isolate	Amalyse activity at 70°C	Cellulase activity at 70°C	Lipase activity at 70°C
THG1	Very good	Very good	Good
THG2	Excellent	Very good	Excellent

Molecular characterization

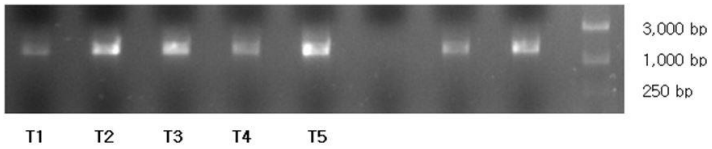

Figure 9 A photograph of amplified 16S rRNA (1.5 kb) of gram negative bacteria using forward primers and reverse from thermophiles isolated from the hot springs of Bhurung tatopani

Table 9 Blast search results of the sequenced isolates from the hot springs of Bhurung tatopani and their close relatives.

Isolate	Blast results (Close relatives)	Percentage Similarity	Genbank Acession number
THG1	*Geobacillus kaustophilus*	99%	KP764939
THG2	*Geobacillus thermoleovorans*	99%	KP764940

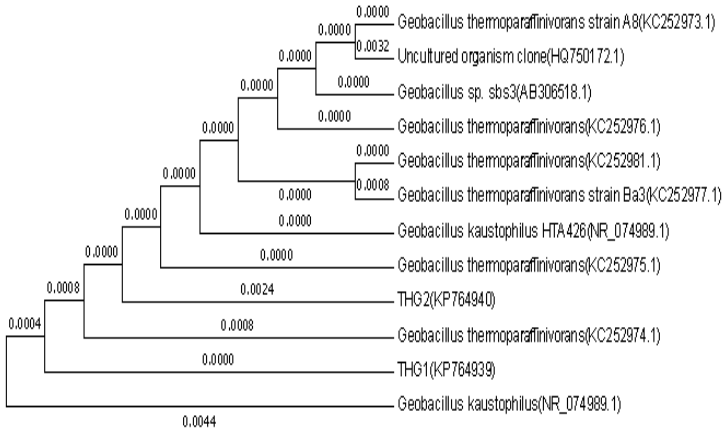

Figure 10 Maximum likelihood distance tree using 16S rRNA sequences of Geobacillus strains isolated from the hot springs of Bhurung tatopani and closest relatives. Bootstrap values as percentages of 1000 replicates are given at branch points; only values >50% are shown.

Fame identification

FAME analysis strains THG1 and THG2 was performed In Royal Life Science, India. They had higher combined levels of C15:0 iso, C15:0 anteiso, C16:0 and C17:0 compared with their closest relatives.

Table 10 Fatty acid composition of strains THG1 and THG2.

Fatty acid	THG1	THG2
12:0	2.07	1.29
11:0 3OH	----	0.27
13:0 iso	0.94	0.80
13:0 anteiso	0.89	0.18
14:0 iso	3.72	1.0
14:0	1.29	1.4
15:1 iso G	----	0.77
15:1 anteiso A	0.52	1.3
15:0 iso	25.73	22.6
15:0 anteiso	12.75	9.43
15:0	----	2.1
16:1 w7c alcohol	1.49	1.09
16:0 N alcohol	1.38	0.19
16:0 iso	9.03	14.96
16:1 w9c	---	21.2
16:0	6.91	11.2
17:1 iso w10c	3.24	1.83
17:0 iso	15.70	18.5
17:0 anteiso	6.77	4.6
17:0	1.74	1.70
18:0 iso	0.75	0.72
18:1 w9c	4.25	1.14
18:0	2.27	3.4
17:0 iso 3OH	0.34	0.13
18:0 10-methyl, TBSA	0.14	0.14
18:0 2OH	0.25	0.16
19:0	0.13	----
20:0	0.18	----
20:0 iso	0.22	----

DISCUSSION

The objectives of this research were to determine the physio-chemical characteristics of the hot springs in Bhurung tatopani, isolate a large number of useful thermophiles from the hot springs, characterize and identify them using morphological, physiological, biochemical and molecular methods and then to screen the isolates for useful enzyme production.

Thermal springs of Bhurung tatopani have extreme physico-chemical conditions (Table 1) suitable for the thermophiles such as *Bacillus*, *Geobacillus* and *Thermus* species. A total of 20 isolates were isolated from the hot springs of Bhurung tatopani, 15 belonged to *Bacillus* genus (paper pertaining this research is already published and can be retrieved online), 2 belonged to *Geobacillus* and 3 belonged to *Thermus* genus thermophiles. Castenholz TYE media was used to increase the chances of isolation of thermophiles. The media was modified by addition of different concentration of sodium chloride and varying the carbon source with glucose, starch and cellulose Also phytagel was used as a gelling agent instead of agar. Comparing to agar, phytagel was required in half amount and gave strong gel after solidifying.

However, the isolates showed a low diversity within morphotypes recovered since most isolates represented the same bacterium i.e. bacillus. This could be attributed to the fact that cultivation is known to capture a small segment of microbial diversity in a given sample. The low diversity of isolates obtained from water sampled at Bhurung hot springs could be due to the fact that very low diversity in hot environments is common. Most of them are distributed in many mesophilic environments, but others have been isolated only from one specific location. It remains possible that there are other strains of thermophiles present in Bhurung tatopani thermal water but since two extreme conditions were imposed at the same time; (high pH and elevated temperature), low diversity was achieved. The combination of two extreme conditions of physio-chemical growth parameters restricts the range at which microorganisms can proliferate more than in a single growth condition.

Microbial enzyme occupies a valuable position in modern biotechnology. The majority of the industrial enzymes known to date have been derived from bacteria and fungi (**Haki and Rakshit, 2003**). Therefore isolation and study of these thermophilic microbes is an vital task for modern scientist. We recorded very significant Amylase and Cellulase activity including others in the isolated strains. The sequence comparison showed that the query sequence coverage was almost 100% and identity was above 99% with the database sequence. What will be the next line of development, is unclear (**Horikoshi, 1999**), but it might be the wider application of enzymes. Alkaline and thermostable enzymes should provide additional uses in various fields of industry, such as chiral-molecule synthesis, biological wood pulping, and more production of sophisticated enzyme detergents. It is expected that in the near future, further aerobic and anaerobic thermophiles with intriguing properties will be isolated from extreme environments using traditional and novel microbial culture techniques and molecular analysis such as metagenomics. These studies will, extend our understanding of the boundaries for conditions under which life can thrive on earth (**Kevbrin *et al.*, 1998**). Subsequently, this will lead to noble theories of how life could have evolved on early earth and whether it could presently or in the future exist in extraterrestrial habitats.

CONCLUSION

The thermal hot springs of Bhurung Tatopani have an average temperature of 65-75ºC and an average pH of 7.5-9 which are extreme conditions suitable for the inhabitation of thermophiles such as *Bacillus*, *Geobacillus* and *Thermus* species. By the use of the Castenholz TYE media and with the help of phytagel as a gelling agent, varying the carbon source (cellulose, xylan, starch and glucose), two *Geobacillus* species were isolated. All The 16S rRNA partial sequences of the these two isolates have been deposited into genebank with genbank Id KP764939 and KP764940 respectively. Detailed biochemical and molecular characterization of the isolated Geobacillus strains was performed and Fatty acid profiling was also performed.

The isolates were thermotolerant, and alkalitolerant because they grew at wide range of temperature (45ºC- 75ºC), pH (5.7-8.0) and NaCl concentration (0-2%) and therefore able to survive in extreme conditions. The thermophiles isolated from the hot springs of Bhurung tatopani were able to produce various thermostable extracellular enzymes like cellulose, lipase, amylase and protease. The sequence information obtained from PCR and sequencing was very good enough for the molecular analysis and identification. For enchancement of the optimum extracellular enzymatic activity further optimization of growth parameters can be performed. Further research is required to design studies that would compare the diversity among thermophiles in different seasons of the year such as the rainy and dry seasons. Different protocols and novel microbial culture techniques should be designed so as to allow the isolation of more diverse genera. Extensive research on the specific secondary metabolites released by thermophiles from the hot springs should be done.

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ISOLATION AND MOLECULAR CHARACTERIZATION OF THREE *BACILLUS* STRAINS FOR THEIR TOLERANCE AGAINST VARIOUS HEAVY METALS

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ABSTRACT

Heavy metal contamination due to natural and anthropogenic sources is a global environmental threat which can produce harmful effects on human health when they are taken up in amounts that cannot be processed by the organism. Technologies involving microbial cells for metal removal and recovery may provide an alternative to conventional methods. In the present study, three cadmium resistant bacteria were isolated from soil collected from industrial area of Faridabad, Haryana, India. Screening of the bacterial isolates for metal resistance against Cd^{2+}, Ni^{2+}, Hg^{2+}, Cu^{+2} and Pb^{2+} was done by determining the minimal inhibitory concentration ranging from 10ppm to 250ppm. Moreover these isolates showed a significant ability to remove 70 to 78% of cadmium. These isolates were identified as *Bacillus sp.263ZY1 (MA5), Bacterium YC-LK-LKJ45 (MB5)* and *Bacillus subtilis strain DHXJ07(MC5)* on the basis of 16S r-RNA gene sequencing. The ability of these microbes to tolerate high concentration of a range of heavy metals provides a scope of use of these bacterial strains for bioremediation of heavy metal from industrial effluent.

Keywords: Heavy metals, *Bacillus* strains, antibiotics sensitivity test, molecular characteristics, bioremediation

INTRODUCTION

The anthropogenic contamination of the environment with heavy metals is a global serious problem. Heavy metals like zinc, cadmium, copper, lead, nickel and mercury have been reported as the most toxic pollutants **(Cameron, 1992)**. Although some heavy metals are essential trace elements, most can be at high concentration, toxic to all forms of life, including microbes, humans and animals. Among the list of heavy metals, the cadmium needs particular awareness because it is recognized as significant pollutant due to its high solubility and toxicity in the water. Cadmium gets released into the environment by various human activities such as mining, smelting, incineration of plastics and batteries, burning of fossil fuels etc **(Tang *et al.*, 2006).** It is toxic at very low exposure levels and has acute and chronic effects on health and environment. Acute exposure to cadmium fumes may cause flu like symptoms including chills, fever, and muscle ache. The long term exposure of these metals result in physical, muscular, neurological degenerative processes that cause Alzheimer's disease (brain disorder), Parkinson's disease (degenerative disease of the brain), muscular dystrophy (progressive skeletal muscle weakness), multiple sclerosis (a nervous system disease that affects brains and spinal cord).Cadmium may also combat with other metals such as zinc and selenium for enclosure into metallo-enzymes and it may compete with calcium for binding sites on regulatory proteins such as calmodulin **(Hu *et al.*, 1998)**. Being non degradable in nature, it stays in circulation once released into the environment. Removal of cadmium from effluents before they are discharged into the environment can be accomplished by processes such as, chemical precipitation, cementation, solvent extraction, reverse osmosis and ion exchange **(Meena and Rajagopal, 2003)**. The physical or chemical methods of heavy metals remediation are neither suitable nor widely accepted for practical applications. A lot of emphasis is being paid to the use of bacteria to remediate heavy metals and it has emerged as one of the alternatives to physicochemical methods. Their use offers economic and ecological benefits, not only as a scientific novelty but also for its potential application in industry **(Chen *et al.*, 2005 and De *et al.*, 2008)**. Some microorganisms have the resistance against the heavy metals and they can grow in the heavy metal rich environment also. To avoid cellular damage caused by metal ions, bacteria evolved mechanisms of metal tolerance. Recent studies shows that the strains (bacteria, yeast and fungi) isolated from contaminated sites possess excellent capability of metal scavenging **(Malik, 2003)**. In the present study the efforts are directed towards the isolation of cadmium resistant bacteria that also shows resistance against other heavy metals and are potential candidates for the comprehensive treatment of metal-rich effluents.

MATERIAL AND METHODS

Sample collection

Soil samples were collected from five different sites of Faridabad industrial area, Haryana, India. All the samples were kept in clean sterile bags and stored at 4^0C.

Isolation and identification of cadmium resistant bacteria

The cadmium resistant bacteria were isolated by standard dilution method. The serially diluted soil samples were spread on the Luria-Bertani agar plates supplemented with 10mM of cadmium as cadmium nitrate. The plates were incubated at 30^0C for 24 hours. The colonies that appeared on the medium supplemented with 10ppm cadmium were considered as cadmium resistant bacteria. Different colony characteristics such as colony color, elevation, shape, margin, texture were determined morphologically distinct colonies was picked and was purified by re-streaking on Luria Bertani medium plates. The isolates were stored on LB agar slants supplemented with 10ppm cadmium in a refrigerator and maintained by regular transfers.

Growth kinetics of bacterial isolates in the presence of cadmium

To study the growth kinetics of the isolates in the presence of heavy metals, LB broth and metal solution were separately autoclaved. The flasks were inoculated with 100µl of overnight grown bacterial culture with different concentration of cadmium (10ppm and 20ppm) and were incubated at 30^0C in rotator shaker. LB broth (without cadmium nitrate) inoculated with the culture served as control. An aliquot of culture was taken out in an oven sterilized tube, at regular intervals of 6, 12, 24, 48, 60, 72, 84, 96, 108 and 120 hours and growth was monitored at regular intervals by measuring the optical density (OD) at 620nm.

Determination of minimal inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of heavy metals was determined by the agar plate dilution method as described by **Malik and Jaiswal (2000)**. Luria Bertani agar medium was prepared and amended with various amounts of heavy

metals (Cd, Pb, Ni, Cu and Hg) to achieve the desired concentration of 10, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 260, 280, and 300ppm. Inoculum of bacterial strains was spread in the metal amended and control plates (without metal). The plates were incubated at 30^0C for 48 hours. The MIC was defined as the lowest concentration of the metals that inhibits the visible growth (number of colonies) of the organisms.

Antibiotic susceptibility

Selected bacterial isolates were tested for susceptibility to 12 different antibiotics(Axiom Laboratories) by the disc diffusion method on Muller Hilton agar (2 g beef extract, 17.50 g Acid hydrolysate of casein, 1.5 g starch, 17 g Agar) plates. The antibiotics tested were: penicillin (20 µg), Co-trimoxazole (25 µg), cephalexin (30 µg), tetracycline (30 µg), cefotaxime (30 µg), ciprofloxacin (5 µg), Levofloxacin (5 µg), linezolid (30 µg), cloxacillin (30 µg), roxithromycin (5 µg), Lincomycin (2 µg) and gentamicin (10 µg). The antibiotics discs were placed on the plates previously seeded with cell suspension. The plates were incubated at 30 0C for 72 h and observed for zones of inhibition.

Isolation of plasmid and electrophoresis

The bacterial isolates were screened for the presence of plasmid DNA using the alkaline lysis method **(Sambrook *et al.*, 1989)**. Plasmid profiles were determined on 0.8% agarose gels prepared in Tris–Borate–EDTA buffer, pH 8.0, stained with ethidium bromide and documented with an Imagemaster digital camera and associated annotation software (Syngene, USA).

Cadmium accumulation and removal assay by bacterial isolates

Selected isolates were grown in LB medium supplemented with 100µg/ml cadmium as cadmium nitrate. The flasks were incubated on a shaker for 120 hours at 30^0C.Cells were harvested at 24 and 48h of incubation by centrifugation (1100xg for 10minutes at 4^0C). Bacterial cell residue was dissolved in 1ml 95% nitric acid mixed well by vortexing and diluted to 10 ml with sterile DDW. Blanks were treated in the same way and analyzed by atomic absorption spectrometry. Cadmium was measured from the supernatant by the atomic absorption spectrometry. Percentage of Cd removal by the bacterial cells from the culture was calculated by taking difference between the initial metal content in the culture media and at the time of sampling **(Sinha and Mukharjee, 2009)**.

16S rRNA sequence and phylogenetic analysis

The metal resistant isolates were identified using 16s rDNA sequencing. Genomic DNA was extracted from the isolates using CTAB method **(Ausubel *et al.*, 1987)** and the quality of the product was checked on 0.8% TBE Agarose gel. PCR amplification of 16S rDNA with forward: 5'-AGHGTBTGHTCMTGNCTCAS-3' and reverse: 5'-TRCGGYTMCCTTGTWHCGACTH-3' primers was carried out using 10ng of genomic DNA. The amplification reactions were performed in a 25µl volume. The standardized conditions for PCR based amplification for each 100µl of reaction mixture were: PCR buffer containing 15mM $MgCl_2$ (10x); 100-200 ng of pure genomic DNA, 1000 pM of each primer, 25µM each of the dNTP's (100µm in a mixture of dNTPs) and 3 units of Taq polymerase. The volume was made up with milli Q water The reaction mixtures were incubated in a thermal cycler (ABI2720, Applied Biosystems) at 94ºC for 5 min and then subjected to 35 cycles consisting of 94ºC for 30s, 55ºC for 30s and 72ºC for 1 min15sec. Finally the mixtures were incubated at 72ºC for 15 min. The amplified product was resolved on 2.0% agarose gel with a constant voltage of 20V for 1h followed by 50V for 3 h. The gels were stained with ethidium bromide and visualized under gel documentation. Sanger sequences were generated using an ABI 3130 Genetic Analyser (Applied biosystem). Phylogenetic analysis was carried out using distance-based phylogeny reconstruction method called "weighted neighbor joining," or "Weighbor" **(Bruno *et al.*, 2000)**. The trees were then validated by bootstrap.

RESULTS AND DISCUSSION

A total of 30 bacterial isolates were initially isolated from soil samples on LB agar plates. Out of thirty, three bacterial isolates were selected on the basis of cultural characteristics such as color, form, margin, and elevation and named as MA5, MB5 and MC5. A detailed result for colonial morphology, biochemical and physiology characteristics has been given in Table 1.

Table 1 Morphological, biochemical and physiological characteristics of selected isolates

Morphological observation	Bacterial isolates		
	MA5	**MB5**	**MC5**
Colony color	Off white	White	Milky white
Gram reaction	Positive	Positive	Positive
Cell shape	Rod	Rod	Rod
Spore formation	positive	Positive	Positive
Elevation	Flat	Flat	Flat
Margin	lobate	lobate	lobate
Biochemical tests			
Catalase test	Positive	Positive	Positive
Starch hydrolysis	Positive	Positive	Positive
H_2S production	Negative	Positive	Negative

Growth kinetics of Bacterial strains in the presence of different concentration of cadmium

Growth kinetics of each of the isolates was studied in the presence of cadmium. Results showed that growth was not considerably affected in the presence of cadmium indicating their resistance to cadmium. MB5 showed maximum growth after 120 hours in the presence of cadmium (Figure1).

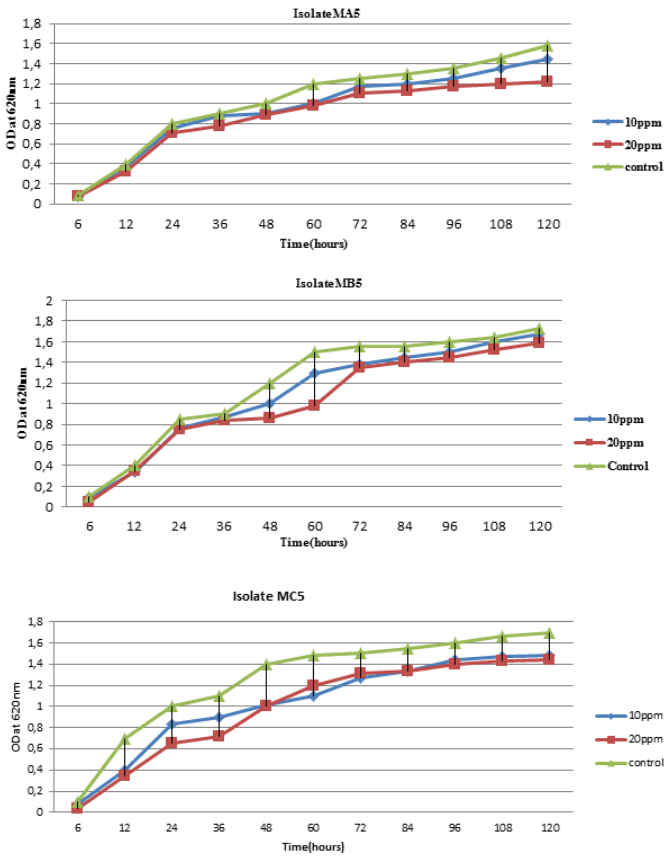

Figure 1 Growth curves of Cadmium resistance bacterial isolates (MA5, MB5 and MC5) in LB medium containing 10ppm and 20ppm concentration.

Minimal Inhibitory Concentration (MIC) of heavy metals

The isolates showed very high degree of resistance to all heavy metals. Minimum inhibitory concentration of Cd for all the isolates was 160ppm. MIC of Hg was 120ppm for MC5, while for the other strains it was 100ppm. MIC of Pb was 180 for all strains. Isolates MA5 and MB5 (100-200ppm) showed to same order of metals toxicity. Among all three bacterial isolates, MC5 showed a very high degree of resistance to heavy metals. The order of heavy metals toxicity towards MC5 was Hg>Pb=Cu>Cd>Ni

Table 2 Minimal inhibitory concentrations (MIC) of various heavy metals to the isolates

Bacterial isolates	Heavy metals(ppm)				
	Cadmium	Lead	Mercury	Copper	Nickel
MA5	160	180	100	180	200
MB5	160	180	100	180	200
MC5	160	180	120	180	200

Antimicrobial susceptibility and Plasmid profile

In order to determine the resistance to antibiotics, the reactions to 12 antibiotics were examined by the disc diffusion method. After incubating for 72 h, the appearance of colonies on plates with antibiotics disc was observed. Depending on the zone of inhibition results were interpreted as susceptible, intermediary susceptible and resistant, as recommended by CLSI. MA5 showed resistance against cefotaxime and levofloxacin. MB5 was resistant to ampicilin, cloxacillin, co-trimoxazole, cephalxin, and cefataxime and sensitive to other antibiotics. MC5 was found resistant to cefotaxime and sensitive to all the other 11 antibiotics.

Table 3 Antibiotic sensitivity for bacterial strains (MA5, MB5, MC5)

Antibiotics	Bacterial isolates		
	MA5	MB5	MC5
Ampicillin(20mcg)	S	R	I
Co-Trimoxazole(25mcg)	I	R	I
Cephalexin(30mcg)	I	R	I
Tetracycline(30mcg)	I	I	I
Cefotaxime(30mcg)	R	R	R
Lincomycin(2mcg)	R	I	I
Ciprofloxacin(5mcg)	S	S	I
Levofloxacin(5mcg)	S	S	S
Linezolid(30mcg)	S	S	S
Cloxacillin(5mcg)	I	R	S
Gentamycin(10mcg)	S	I	S
Roxithromycin(15mcg)	S	S	S

R-resistant, I-Intermediate resistance, S-sensitive

Isolation of plasmid and electrophoresis

All the three bacterial isolates were screened for the presence of plasmid. Plasmid profile (Figure 3) indicates the presence of a mega plasmid (more than 10,000 bp).

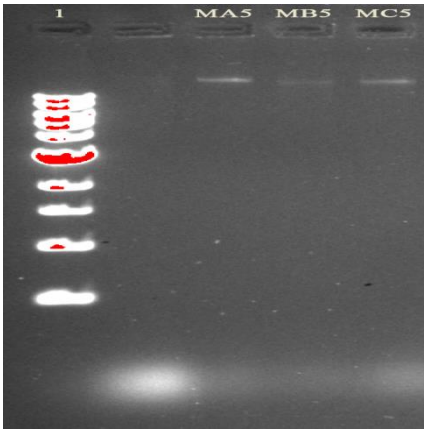

Figure 3 Plasmid DNA was extracted and separated by agarose gel electrophoresis (DNA ladder in lane 1); MA5 is loaded in lane3, MB5 in lane 4, MC5 in lane5.

Cadmium removal assay

The isolates were examined for their ability to remediate the cadmium from aqueous solution. It was observed that the isolates could remove upto 78% of the Cd from medium amended with 100ppm cadmium (Tab 4). Isolate MA5 showed maximum removal of Cd (74 %and 78%), after 24 and 48hours of incubation respectively and isolate MB5 could efficiently remove 70% and 72% of Cd after 24 and 48hours of incubation respectively. In case of MC5, the concentration of Cd was reduced to 74% from an initial concentration of 100ppm in 48 hours.

Table 4 Percentage removal of cadmium by bacterial isolates from medium with initial concentration of 100 ppm cadmium after 24 and 48hours of incubation at 30^0C.

Bacterial isolates	Cadmium removal (%)	
	After 24h	After 48h
MA5	74	78
MB5	70	72
MC5	72	74

16S r-RNA sequence and phylogenetic analysis

The genomic DNA (Figure 4) was isolated and amplified with 16S rRNA specific primers. The length of the amplified DNA fragments is about 1.5bp (Figure 5). Sequence analysis of the DNA fragments was performed and compared against the GenBank database using the NCBI Blast program. Selected sequences of other microorganisms with greatest similarity to the 16SrRNA sequences of bacterial isolates were extracted from the nucleotide sequence databases and deposited in GenBank under accession number of KU513823, KU513824, KU513825 for MA5, MB5and MC5 respectively (Tab 5). These 16S rRNA sequences were aligned by MUSLE for construction of phylogenetic tree using neighbor joining method **(Saitou and Nei 1987)**. Evolutionary analyses were conducted in MEGA7 **(Kumar *et al.*, 2015).**

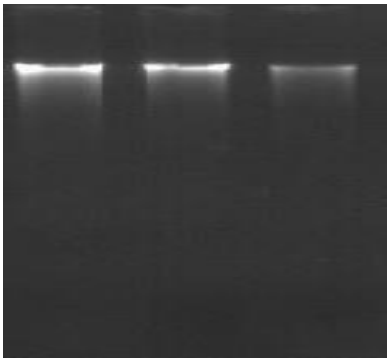

Figure 4 Extraction of Genomic DNA from Bacterial sample using the Bacterial Genomic DNA Isolation Kit (RKN15).

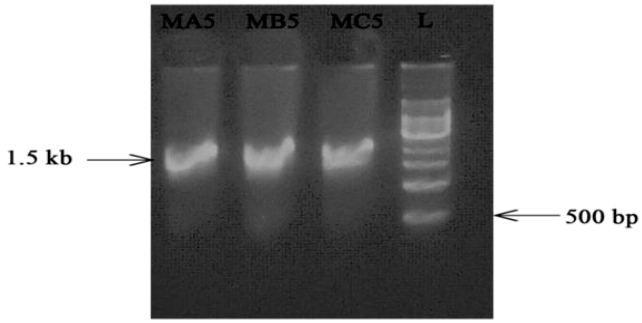

Figure 5 PCR amplification of 16s rDNA fragment from Bacterial sample. The size of PCR amplified product is ~1.5kb.

Table 5 Molecular identification of the isolates based on partial 16S rDNA analysis

Bacterial isolates	Bacterial strain showing maximum homology	Identity (%)	GenBank accession No.
MA5	*Bacillus sp. 263ZY1*	99%	KU513823
MB5	*Bacterium YC-LK-LKJ45*	99%	KU513824
MC5	*Bacillus subtilis strain DHXJ07*	99%	KU513825

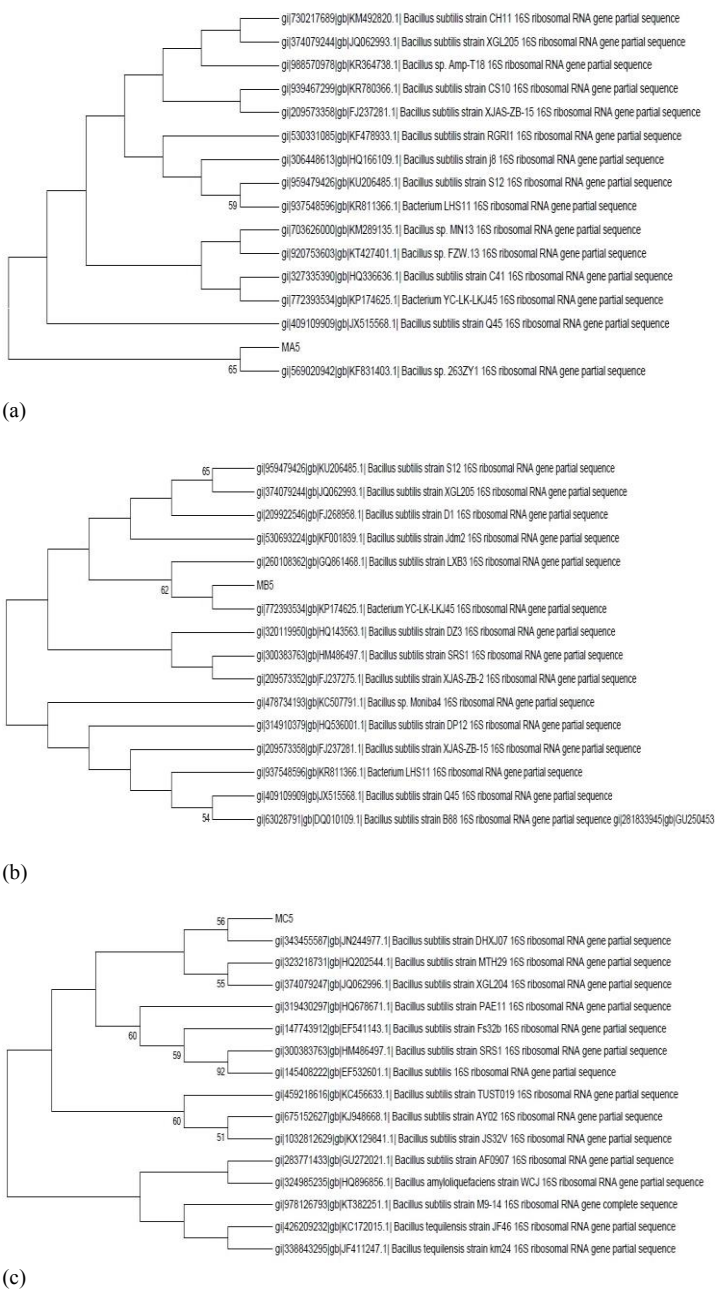

Figure 6 Phylogenetic analysis of the isolates based on 16S rDNA sequence analysis. (a) Phylogenetic tree of isolate MA5, (b) phylogenetic tree of isolate MB5, and (c) phylogenetic tree of isolate MC5.

DISCUSSION

Metal polluted environment contains microorganisms which have adapted to toxic concentrations of heavy metal. Such a microbial population is resilient to metal toxicity by various mechanisms involving bioaccumulation and bioadsorbtion, oxidation and reduction, methylation and demethylation and extrusion of toxic ions by efflux systems **(Bolton and Gorby, 1995)**. These microorganisms can potentially remove heavy metals from the polluted environment. Many bacterial species, both gram positive and gram negative, have been implicated in bioremediation studies. In the present study, three cadmium resistant bacteria were isolated from industrial soil samples which tolerated high concentrations of Cd^{+2}, Pb^{+2}, Ni^{+2}, Cu^{+2} and Hg^{+2}.These isolates were also found to be resistant to many antibiotics. Since there is a documented association between metal resistance and antibiotic resistance and the genes conferring the same are thought to reside on a plasmid, all the three bacterial isolates were screened for the presence of plasmids and it was found that all the isolates harboured the plasmid. Many studies indicate a close association between the types and levels of metal contamination and specific patterns of antibiotic resistance. The mechanisms that underlie this co-selection process include co-resistance and cross-resistance **(Baker-Austin *et al.*, 2006)**. The selected isolates were found to be resistant to a range of heavy metals demonstrating that environmental bacteria may adapt to their ecological conditions and may have been selected for certain metal tolerance mechanisms. Such bacterial strains may be potential candidates for their simultaneous removal from wastes. Several studies on application of growing microbial cells for metal scavenging have been reported **(Malik, 2004)**. Under lab conditions, these isolates were able to remove upto 70-78% cadmium. A slight difference in phenotypic characteristics was observed among three bacterial isolates, suggesting the presence of different species. The morphological characters and biochemical attributes of isolates MA5, MB5 and MC5 provide evidence to their resemblance with *Bacillus* genera. Further molecular characterization was done to confirm identification. The 16S-rDNA sequencing confirmed identification of MA5, MB5 and MC5 as *Bacillus sp.263ZY1, Bacterium YC-LK-LKJ45* and *Bacillus subtilis strain DHXJ0,* respectively.

Many studies have implicated *Bacillus spp.* in bioremediation of heavy metals. *Bacillus safensis* strain was found to tolerate cadmium up to the concentration of 80 ppm and the reduction and absorption of cadmium was 83.5, 39% and 98.10, 92% at 40 and 60 ppm of cadmium, respectively at pH 7. **Khosro *et al.* (2011)** used different *Bacillus* strains for the removal of heavy metals. The results revealed that maximum cadmium accumulation were 7.3mol/g biomass for *B.licheniformis,* 10.7 mol/g biomass for *B.cereus*, 9.5 mol/g biomass for *B. subtilis* and 7.2 mol/g biomass for *B. amyloliquefaciens*. In another study, *Bacillus subtilis* isolated from industrial effluent soil showed maximum accumulation at 200µg/ml and showed saturation above that concentration (250 µg/ml) **(Gayathramma *et al.*, 2013)**. Significant reduction of soluble Cd was observed during growth of plasmid-bearing *Bacillus* strain H9 and *Pseudomonas* strain **H1 (Roane and Pepper, 2000)**.

The environmental contamination arising primarily from agricultural and industrial sources, as a result of the increase in population, industrial activities, and the modernization of agricultural practices, such as the increasingly widespread use of pesticides, resulting in an increase of the amount of effluents thrown into the air, water, and soil, and have consequences on food quality and human health which are very serious worldwide problems. Bacteria have been so continuously exposed to heavy metal contaminants of the environment that they have developed genetically determined resistance systems against heavy metal toxicity. Contaminated environments like those in the vicinity of industries or industrial dump grounds accumulate a heavy burden of toxic metal ions, organic wastes and antibiotics. A large number of bacterial isolates from these areas have depicted a pronounced capability of processing and resisting toxic industrial wastes. All the three bacteria isolated in this study show a high efficiency in removal of Cd from the medium. They accumulated a significant amount of Cd within 24h. These three strains were belong to *Bacillus* spp. Cadmium uptake is reported to be through an ATPase pump in Gram positive bacteria and by the action of proton-cation anti-porters in Gram negative bacteria **(Nies and Silver1995)**. These capabilities of the selected strains make them suitable for bioremediation of contaminated environment.

CONCLUSION

The present investigation was designed to isolate cadmium resistant bacteria which possessed multiple resistances to various heavy metals and consequently could be used for the simultaneous removal of more than one heavy metal from a contaminated environment. The capability of microbial strains to grow in the presence of heavy metals would be helpful in the waste water treatment. In the present study, bacterial isolates belonging to genera *Bacillus*, showing high degree of resistance to various heavy metals was isolated from soil. These strains were capable of removing upto 78 % of cadmium, signifying that these *Bacillus* strains can be efficiently used for bioremediation and removal of cadmium present in polluted water with minimum cost and high efficiency.

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ISOLATION AND IDENTIFICATION OF *Elizabethkingia meningoseptica* FROM DISEASED AFRICAN CATFISH *Clarias gariepinus*

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ABSTRACT

Two isolates of *Elizabethkingia meningoseptica* were successfully isolated from kidney and skin tissue of diseased African Catfish (*Clarias gariepinus*) in Malaysia. The percentage similarity of both physical and biochemical characteristics for the isolates from kidney (K1) and skin samples (S1) as determined by BBL-Crystal and API 20E were 99 % and 95.4%, respectively. Furthermore, both isolates were identified *via* 16S rRNA gene sequences and showed more than 97% homology to sequences deposited in GenBank. The API ZYM results were analogous for both strains, with only minor quantitative variations. However, the isolates from kidney sample (K1) showed higher levels of enzymatic activity reaction towards esterase lipase and leucine arylamidase. Moreover, the enzymatic activity of α-galactosidase was detected at low level in kidney isolate and absence in skin isolate.

Keywords: *Elizabethkingia meningoseptica*, polymerase chain reaction, *Clarias gariepinus*, BBL-Crystal, Enzymatic activity

INTRODUCTION

Bacteria display diverse morphologies when they are grown on different culture media. Thus, bacterial species identification is determined using morphological, biochemical and physiological investigations. For instance, Gram staining, which is based on the amount of peptidoglycan in the bacteria cell wall, as well as several series of biochemical tests are classical method for bacterial characterization **(Pollack *et al*., 2008)**. Nowadays, molecular methods are used to identify an organism **(Jacobs & Chenia *et al*., 2011)**.
Elizabethkingia meningoseptica has been recently isolated from diseased American bullfrog in Malaysia **(Ransangan *et al*., 2013)**; however, never has it been isolated from *Clarias gariepinus* . *E. meningoseptica* is the etiological agent of disease in South African clawed frog (*Xenopus laevis*) and leopard frog (*Rana pipiens*) **(Xie *et al*., 2009)**. This bacterium is also a significant human pathogen, where it has been found to cause pneumonia, meningitis, postoperative bacteraemia in adults, fatal necrotizing fasciitis in diabetic patients, and meningitis in premature neonates **(Bloch *et al., 1997;* Gupta *et al*., 1997; Lee *et al*., 2006; Lee *et al*., 2008).**
Presently, *E. meningoseptica* infections in aquaculture are unknown **(Kirby *et al*., 2004)**. Therefore, the present study was aimed to isolate and identify *E. meningoseptica* from diseased catfish (*Clarias gariepinus*). This is the first study to isolates *E. meningoseptica* from fish in Malaysia.

MATERIALS AND METHODS

Bacterial Isolation

Diseased *Clarias gariepinus* catfish were collected from a local fish farm located at Marang River Terengganu, Malaysia (05°12'N, 103°13'E). The fish weighed from 350 to 400 g. The fish were dissected according to **Wilson *et al* (2009)** which were performed using standard methods **(Buller *et al*., 2004)**. Shieh agar supplemented with tobramycin (1 mg ml^{-1}), enriched Anacker and Ordal's agar (EAOA) supplemented with polymyxin B (10 U ml^{-1}) and neomycin (5 µg ml^{-1}) were prepared. Bacteriological swabs were aseptically taken from the skin lesions, liver and kidney of catfish. Incubation was done at 28°C for 48 h. The presence of flexirubin-type pigments in bacterial colony was determined using the KOH method **(Bernardet *et al*., 2002)**. Shieh agar and Anacker and Ordal's agar (EAOA) were selected to isolate and maintain the bacteria. The pure cultures were kept in enriched Anacker and Ordal's broth (EAOB) supplemented with 20% glycerol at -80°C.

Bacterial Identification

The Gram reaction was performed as described by **Gerhardt *et al* (1994).**The bacterial colony morphology was determined after 48 h of incubation at 28°C. The bacterial isolates were characterized biochemically by standard biochemical tests (e.g. Indole Test, Methyl Red Test, Voges Proskauer Test, Urease Test, Catalase Test, Oxidase Test, Gelatin Hydrolysis, Oxidative fermentative utilization of glucose, Simmons citrate test, H_2S production, Casein hydrolysis, Acid and gas production from sugars, phenylalanine test, β-galactosidase test, lysine decarboxylase and arginine dehydrolase, and Starch Hydrolysis). Physiological temperature tolerance tests at 5°C, 28°C, 37°C, 42°C, and salt tolerance at different concentrations (0.5 %, 1.5 %, 3 %, 6 %, 8 %, 10 %) of NaCl (w/v) were also performed according to **Cappuccino & Sherman *et al* (1996)**; **Prescott *et al* (2005)**; **Pollack *et al* (2008)**. Flexirubin-type pigment was detected with 20 % KOH according to the method of **Fautz & Reichenbach *et al* (1980)**. The isolates were subjected to hemolysis test as described by **Pavlov *et al* (2004).** The isolates were further biochemically identified using BBL-Crystal™Enteric/Non Fermenter Identification System Kit (USA), API 20E, and API ZYM System (bioMerieux, France) according to the manufacturers' instructions.

16S rRNA Identification

The DNA was extracted from the isolates using Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instruction. 16S rRNA gene PCR was carried out using published universal bacterial primers (5'-GAT TAG ATA CCC TGG TAG TCC AC-3' and 5'-CCC GGG AAC GTA TTC ACC G-3') to generate a fragment approximately 610 bp in length according to **Kim *et al* (2003)** and **Sun *et al* (2008)** using MasterCycler Personal (Eppendoft, Germany). The PCR was performed with minor modification. The reaction began with an initial denaturation step of 96°C for 2 min followed by 35 cycles of 96°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The reaction was terminated by an extension step of 72°C for 10 min. Negative control with sterile distilled was included in PCR. The PCR products were analyzed using 1.5% agarose gel (molecular grade) electrophoresis, stained with ethidium bromide, and viewed under ultraviolet light. The PCR products were purified using Gene

MATRIX PCR/DNA Clean-Up DNA Purification Kit (EURx, Poland). The DNA sequences deduced were subjected to standard basic local alignment search tool (BLAST) analysis at National Center for Biotechnology Information **(NCBI, 2012)** to determine the identity of the isolates.

RESULTS

Bacterial Isolation

The diseased fish showed symptoms of increased respiration and lethargy, skin lesions such as white discoloration, shallow hemorrhagic ulcers and deep ulcers with exposed underlying muscle. Some fish showed marked haemorrhages on the base of the fins and vents. Others were dropsy, showed kidney congestion and enlargement, pale-coloured of liver and gills, and/or gall-bladder enlargement with the accumulation of yellowish fluid in the body cavity **(Figure 1).** Bacterial isolates were obtained from kidney (K1) and skin lesion (S1) that were grown on Shieh and enriched with Anacker and Ordal's agar (EAOA). Colonies were small (1 to 2 mm diameter), smooth surface, convex, circular, and shiny with complete edges on EAOA and Shieh agar. Colonies were yellowish and creamy in colour on EAOA agar Shieh agar, respectively. Bacteria were Gram negative, non-motile, straight, single short rods approx. 2 µm in length and 1 µm in width **(Figure 2)**. They were then subjected to identification using conventional, commercial kits, and 16S rRNA PCR.

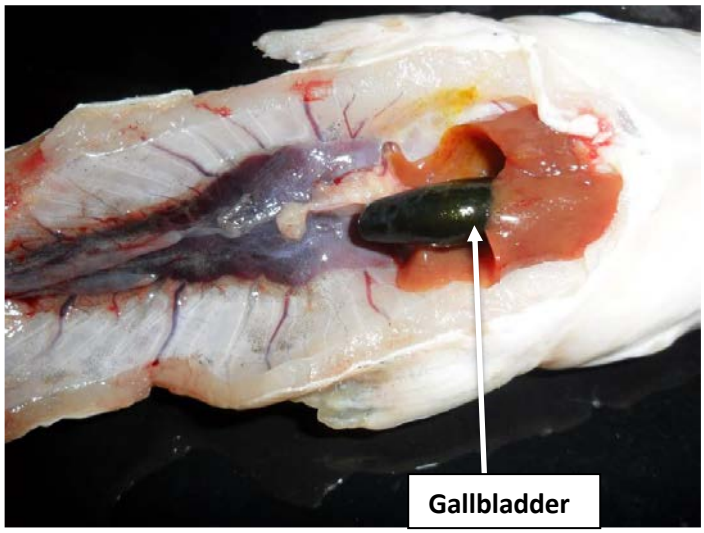

Figure 1 Catfish, *Clarias gariepinus,* infected with *Elizabethkingia meningoseptica* showing enlargement of the gallbladder

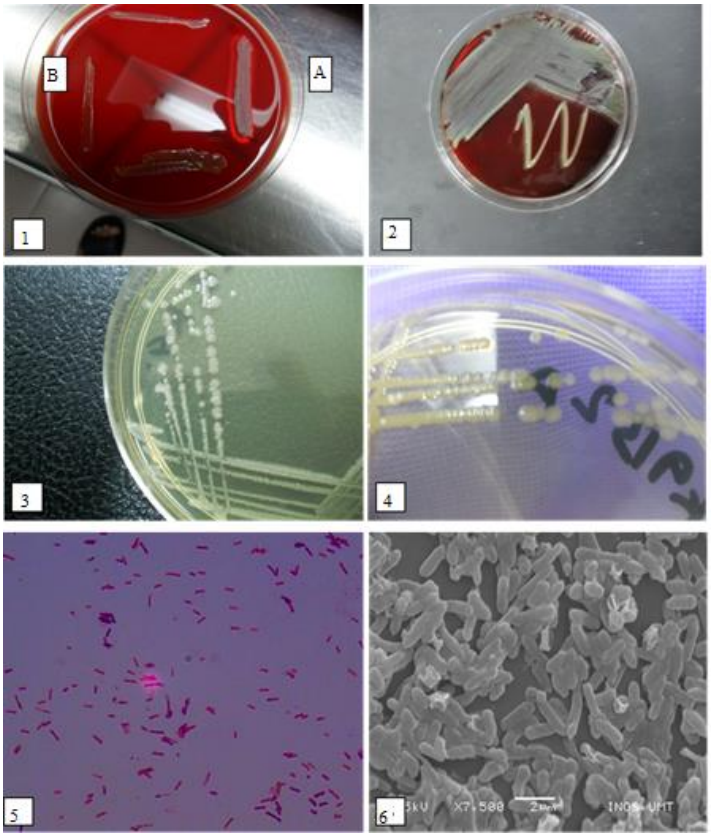

Figure 2 Morphology of *Elizabethkingia meningoseptica.* Blood agar showing haemolytic activity of *E. meningoseptica* (A): Kidney isolates showing beta hemolysis. (B): Skin isolates showing gamma hemolysis; **2-***E. meningoseptica,* skin isolated growth on blood agar showing gamma haemolytic activity; **3-**(A) Colony morphologies displayed by *E. meningoseptica* isolate on Shieh agar; creamy-white, small shiny convex colonies with smooth edges; **4-**(B) Colony morphologies displayed by *E. meningoseptica* isolate on (EAOA); pale yellow, larger convex colonies with smooth edges; **5-** Light microscope image of Gram-negative staining reaction displayed by *E.meningoseptica* isolates (×1000 magnification); **6-** Scanning Electron Microscope (SEM) of *E.meningoseptica*

Bacterial Identification

Physical and biochemical characteristics of the isolates were carried out according to standard protocol. BBL-Crystal ™ Enteric/Non fermenter Identification System kit (USA), API 20E and API ZYM systems (bioMerieux, France) were used for the purpose. Phenotypic characteristics of kidney isolate and skin isolate are shown in Chyba! Nenašiel sa žiaden zdroj odkazov..

Table 1 Phenotypic characteristics of *E. meningoseptica* isolates

Characteristics	K1	S1
Gram Stain	-	-
Indole production	+	+
Methyl red	-	-
Voges-proskauer	-	-
H_2S production	-	+
Ornithine decarboxylase	-	-
Arginine dihydrolase	+	+
Motility	-	-
Oxidase	+	+
Catalase	+	+
Blood hemolysis	B	γ
Lipid hydrolysis	-	-
Gelatine hydrolysis	+	-
Starch hydrolysis	+	+
Casein hydrolysis	+	+
Utilization		
Glucose,acid	+	+
Lactose,acid	-	-
Maltose,acid	+	+
Raffinose,acid	-	-
Trehalose, acid	+	+
Xylose,acid	-	-

Characteristics	K1	S1
Mannose,acid	-	-
Sucrose,acid	-	-
Rhamnose,acid	-	-
Sorbitol,acid	-	-
Mannitol,acid	-	-
Inositol,acid	-	-
Melibiose	-	-
Arabinose	-	-
Adonitol	-	-
Galactose	-	-
p-nitrophenyl phosphate	+	+
p-nitrophenyl α-β-glucoside	+	+
p-nitrophenyl β-galactoside	-	+
Prolinenotroanilide	+	+
p-nitrophenylbis-phosphate	+	-
p-nitrophenylxyloside	-	-
p-nitrophenyl α-arabinoside	-	+
p-nitrophenylphosphorylcholine	+	-
p-nitrophenly β-glucuronide	-	-
p-nitrophenyl-N-acetyl glucosaminide	+	-
γ-L-glutamyl p-nitroanilide	+	+
Esculin	+	+
p-nitro-DL-phenylalanine	-	-
Urea	-	-
Glycine	+	+
Citrate	-	-
Malonate	-	-
Tetrazolium	+	+
Arginine	-	-
Lysine	+	-
Growth at NaCl		
0.5 %	+	+
1.5 %	+	+
3 %	-	-
6 %	-	-
8 %	-	-
10 %	-	-
Growth at		
5o C	-	-
28o C	+	+
37o C	+	+
42o C	-	-

* K1:*E. meningoseptica* isolate from the kidney of catfish (*Clarias gariepinus*); S1:*E. meningoseptica* isolate from the skin of catfish (*Clarias gariepinus*)

The isolates produced indole, but scored negative in the Methyl red-Voges – proskauer and hydrogen sulfide tests. Additionally, *Elizabethkingia meningoseptica* strains isolated from kidney showed to be β-hemolytic on blood agar. *E. meningoseptica* strains isolated from skin showed to be γ- hemolytic activity **(**Chyba! Nenašiel sa žiaden zdroj odkazov.**)**.

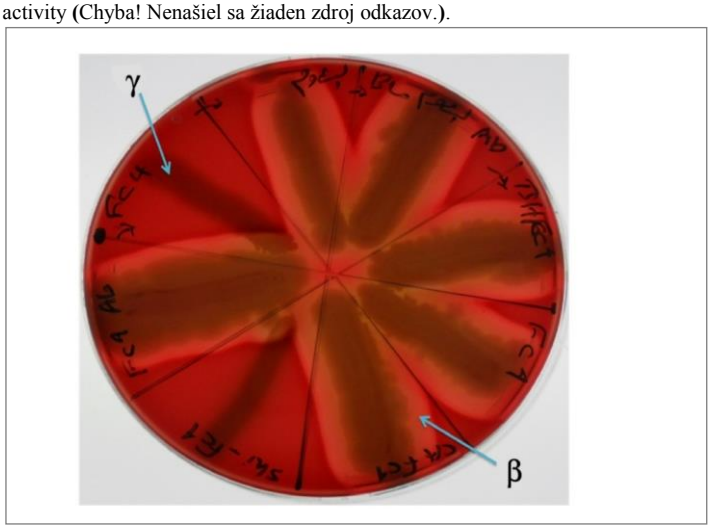

Figure 3 Isolate from kidney showing beta haemolytic activity (β) while Isolate from skin showing gamma haemolytic activity (γ)

In physiological test, both isolates grew at 28˚C and 37˚C. In the salt tolerance test, both isolates grew on media supplemented with 0.5 % and 1.5 % NaCl media. BBL results showed that isolates K1 and S1 were 99 % similar to *E. meningoseptica* **(**
). On the other hand, API 20E identified isolates K1 and S1 as *E. meningoseptica* with 95.4% similarity **(**Chyba! Nenašiel sa žiaden zdroj odkazov.**)**.

Table 2 Biochemical characteristics of *Elizabethkingia meningoseptica* based on BBL-Crystal system

Test	Isolate	
	K1	S1
Oxidase	+	+
Indole	+	+
Arabinose	-	-
Mannose	-	-
Sucrose	-	-
Melibiose	-	-
Rhamnose	-	-
Sorbitol	-	-
Mannitol	-	-
Adonitol	-	-
Galactose	-	-
Inositol	-	-
p-nitrophenyl phosphate	+	+
p-nitrophenyl α-β-glucoside	+	+
p-nitrophenyl β-galactoside	-	+
Prolinenitroanilide	+	+
p-nitrophenylbis-phosphate	+	-
p-nitrophenylxyloside	-	-
p-nitrophenyl α-arabinoside	-	+
p-nitrophenylphosphorylcholine	+	-
p-nitrophenly β-glucuronide	-	-
p-nitrophenyl-N-acetyl glucosaminide	+	-
γ-L-glutamyl p-nitroanilide	+	+
Esculin	+	+
p-nitro-DL-phenylalanine	-	-
Urea	-	-
Glycine	+	+
Citrate	-	-
Malonate	-	-
Tetrazolium	+	+
Arginine	-	-
Lysine	+	-

* K1: kidney; S1: skin; +: positive - : negative

Table 3 Identification of *Elizabethkingia meningoseptica* based on API20E

Tests	Substrate	Stain Reaction	
		K1	S1
ONPG	ONPG	+	+
ADH	Arginine	-	-
LDC	Lysine	-	-
ODC	Ornithine	-	-
CIT	Citrate	+	+
H2S	Na thiosulfate	-	-
URE	Urea	+	+
TDA	Tryptophan	-	-
IND	Tryptophan	+	+
VP	Na pyruvate	-	-
GEL	charcoal gelatin	+	-
GLU	glucose	-	-
MAN	mannitol	-	-
INO	inositol	-	-
SOR	sorbitol	-	-
RHA	rhamnose	-	-
SAC	sucrose	-	-
MEL	melibiose	-	+
AMY	amygdalin	+	-
ARA	arabinose	-	-
OX	Oxidase	+	+

* K1: kidney; S1: skin; +: positive - : negative

The characteristics of each isolate tested on the API ZYM system are listed in Chyba! Nenašiel sa žiaden zdroj odkazov. and

Table 5 Hydrolytic enzymes and their substrates assayed based on API ZYM test. The API ZYM results were very similar for both strains, with only minor qualitative variations. Both isolates showed a low level of reactions to the enzymes; Esterase (C4), Lipase (C14), Cystinearylamidase, Trypsin, α-chymotrypsin, β-galactosidase, α-glucosidase, β-glucosidase and N-acetyl-β-glucosaminidase. They were negative for β-glucuronidase and α-mannosidase, but there were higher levels of enzymatic activities such as alkaline phosphatase, valinearylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. However, isolate K1 showed higher levels of enzymatic activity reaction towards esterase lipase and leucine arylamidase. Furthermore, the enzymatic activity of α-galactosidase was detected at low level in kidney isolate and absence in skin isolate.

Table 4 Enzymatic profiles for *Elizabethkingia meningoseptica* based on API ZYM

Enzymes/Isolates	Substrate	K1	S1
Control		-	-
Alkaline phosphatase	2-naphtylophosphate	+/s	+/s
Esterase (C4)	2-naphtylbutyrate	+/w	+/w
Esterase Lipase (C8)	2-naphtylcapylate	+/s	+/w
Lipase (C14)	2-naphtylmyristate	+/w	+/w
Leucinearylamidase	L-leucyl-2-naphthylamide	+/s	+/w
Valinearylamidase	L-leucyl-2-naphtylamide	+/s	+/s
Cystinearylamidase	L-cystyl-2-naphthylamide	+/w	+/w
Trypsin	N-benzoyl-DL-arrginine-2-naphthylamide	+/w	+/w
α-chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide	+/w	+/w
Acid phosphatase	2-naphthylphosphate	+/s	+/s
Naphthol-AS-BIphosphohydrolase	Naphthyl-AS-BI-phosphate	+/s	+/s
α-galactosidase	6-Br2-naphthyl- D-galactopyranoside	+/w	-
β-galactosidase	2-naphthyl- D-galactopyranoside	+/w	+/w
β-glucuronidase	Naphthol-AS-BI- D-glucuronide	-	-
α-glucosidase	2-naphthylyl- D-glucopyranoside	+/w	+/w
β-glucosidase	6-Br-2-naphthyl- D-glukopyranoside	+/w	+/w
N-acetyl-β-glucosaminidase	1-naphthyl-N-acetylo- D glucosaminide	+/w	+/w
α-mannosidase	6-Br-2-naphthyl- D-mannopyranoside	-	-
α-fucosidase	2-naphthyl- -L-fukopiranoza	-	-

***** K1: kidney; S1: skin; +: positive; - : negative; W: weak; S: strong

Table 5 Hydrolytic enzymes and their substrates assayed based on API ZYM test

Enzymes/Isolates	K1	S1	Sum
Control	0	0	0
Alkaline phosphatase	5	4	9
Esterase (C4)	3	3	6
Esterase Lipase (C8)	5	3	8
Lipase (C14)	2	2	4
Leucinearylamidase	5	2	7
Valinearylamidase	4	4	8
Cystinearylamidase	3	2	5
Trypsin	1	2	3
α-chymotrypsin	1	1	2
Acid phosphatase	5	5	10
Naphthol-AS-BIphosphohydrolase	4	5	9
α-galactosidase	2	0	2
β-galactosidase	1	1	2
β-glucuronidase	0	0	0
α-glucosidase	1	1	2
β-glucosidase	1	1	2
N-acetyl- β-glucosaminidase	2	3	5
α-mannosidase	0	0	0
α-fucosidase	0	0	0
Sum	45	39	84

***** Numbers indicate the relative expression of enzymes which is estimated from the color intensity as strong (4-5), weak (1-3), or negative (0). Mean values were rounded up.

Both isolates had a similar enzymatic profile but kidney isolate had a weak positive reaction for α-galactosidase as compared to skin **(Table 5)**. Both isolates were identified using 16S rRNA gene sequences analysis. PCR amplification of 16S rRNA gene sequences of isolates is presented in **Figure 4**. All 16S rRNA gene sequences showed more than 97% homology to sequences deposited in GenBank. The isolates were identified as *E. meningoseptica*,
16S rRNA sequence alignments of *Elizabethkingia meningoseptica* bacteria isolated from kidney and skin isolates of diseased catfish (*Clarias gariepinus*) are shown in Appendix A and Appendix D, respectively. Genbank accession 16S rRNA S1 (skin) strain and 16S rRNA K1 (kidney) strain of *Elizabethkingia meningoseptica*are highlighted in Appendix H and Appendix G, respectively.

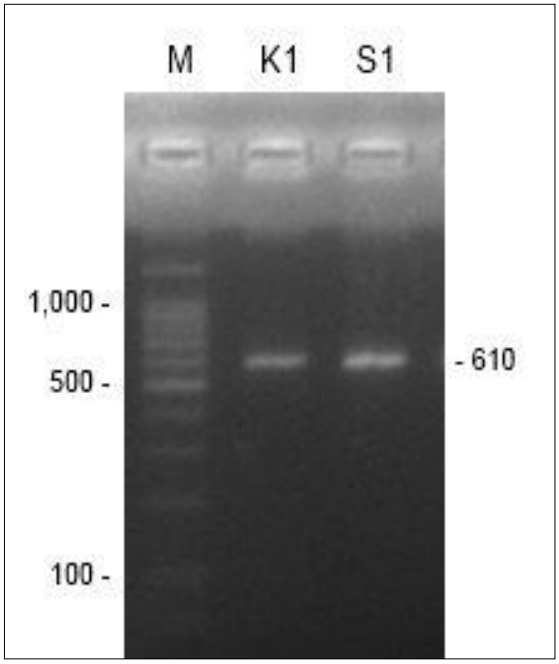

Figure 4 Electrophoresis on 1.5% agarose gel of universal PCR products of 16S rRNA gene of *Elizabethkingia meningoseptica* isolated from kidney (Lane K1) and *Elizabethkingia meningoseptica* isolated from skin (Lane S1) amplified at 610 bp with standard molecular weight marker (M 1 kb)
***** Lane M: 100 bp marker; Lane 2: K1 isolate; Lane 3: S1 isolate

All 16S rRNA gene sequences of the 2 bacterial isolates showed more than 97% homology to sequences were deposited in GenBank with the accession number for kidney isolate BankIt 1604905 KC757123 and for skin isolate BankIt 1611391KC757124. The isolates were identified as *E. meningoseptica*. Blast result and electropherogram are shown in respective appendices (Appendix A, B, C & D).

DISCUSSION

Recently, Gram-negative, non-fermentative bacteria are increasingly implicated in human disease **(Gautam *et al*., 2011)**. In 1959, American bacteriologist Elizabeth O. King was examining unclassified bacteria associated with meningitis in infants when she isolated an organism that she named *Flavobacterium meningosepticum*. In 1994, *Flavobacterium meningosepticum* was reclassified in the genus *Chryseobacterium* and was named *Chryseobacteriummeningosepticum*. In 2005, a 16S rRNA phylogenetic tree illustrated that *C. meningosepticum* and *C. miricola* were related; however, dissimilar to the rest of the Chryseobacteria family. They were then placed in a new genus: *Elizabethkingia* **(Kim *et al*., 2005).**

Elizabethkingia meningoseptica are opportunistic pathogens in both veterinary and human infections. Their pathogenicity and association with food spoilage has caused some economic losses in the aquaculture and food industries. *E. meningoseptica* causes equipment-associated infections in immuno-compromised humans **(Jacobs & Chenia *et al*., 2011**; **Kirby *et al*., 2004; Michel *et al*., 2005)**. *E. meningoseptica* was formerly reported as the etiological agent of disease in South African clawed frogs (*Xenopus laevis*) and leopard frogs (*Rana pipiens*) **(Xie *et al*.,2009)**. In the present research, an appropriate approach was used to analyse bacterial isolates from diseased catfish. Moreover, the present study is the first report of *Elizabethkingia meningoseptica* isolated from catfish in Malaysia. All isolates were identified as *E. meningoseptica* by biochemical and physiological tests, commercial identification kit, and were conformed based on their 16S rRNA gene sequences. Most of the biochemical characteristics of these strains were consistent with *E. meningoseptica* **(Decostere *et al*., 1998**; **Kim *et al*., 2005)**, with the exception of acid being produced from xylose from a few isolates especially those isolated from skin. Based on previous research, the study was carried out to isolate the bacteria from fish. Primary isolates were placed on Shieh agar enriched with a 1 µg ml^{-1}tobramycine **(Decostere *et al*., 1998)**, and the isolates were later enriched on Enriched Anacker and Ordal's agar (EAOA) supplemented with polymyxin B (10 U ml^{-1}) and neomycin (5 µg ml^{-1}) **(Plumb *et al*., 1999)** . In the study of **Bernardet *et al* (2005),** it was reported that Shieh agar and enriched Anacker and Ordal's agar (EAOA) performed well for growing bacteria. On the other hand, these isolates also grew on trypticase soy agar (TSA) at 28˚C for 48 h. The colonies were pale yellow or creamy-white in these entire medium. In the present study, both isolates showed no growth at 5˚C or 42˚C, these finding are in accordance to **Kim *et al* (2005)**, in that good growth was observed on trypticase soy agar (TSA) and nutrient agar at 28˚C to 37˚C. In order to purify the colonies, 2 or 3 replications were performed. For long-term storage, the cultures were kept in Enriched Anacker and Ordal's broth (EAOB) supplemented with 20% glycerol and stored at -80^0 C **(Jacobs & Chenia *et al*., 2011)**. According to **Fijan *et al* (1969)**, addition of polymyxin B (10 U ml^{-1}) and neomycin (5 µg ml^{-1}) to Cytophaga agar could increase the number of *Chondrococcus columnaris* colonies and suppress the growth of other bacteria. Moreover, **Flint *et al* (1985)** showed that inclusion of 10 µg ml-1 kanamycin in nutrient agar could increase the percentage of yellow pigmented colonies from 15% to 55%. In another study, **Decostere *et al* (1998)** revealed that *Flavobacterium columnare* was inhibited by 1 µg ml^{-1} of tobramycin than other fish-associated bacteria.

The results of this study demonstrated that *E. meningoseptica* colonies exhibit the following features: pale yellow to creamy color, round colonies 1 to 2 mm in diameter, this finding is in agreement with previous report by **Kim *et al* (2005)** where *E. meningoseptica* colonies are smooth and fairly large, white yellowish, colonies diameter of 1.0 to 1.5 mm grown on Shieh and Enriched Anacker and Ordal's agar (EAOA). In our study, *E. meningoseptica* was Gram negative, straight rods, non-motile; catalase, and oxidase positive comparable to reports by **Sarma *et al* (2011)**. In addition, *E. meningoseptica* could not ferment the carbohydrate (sugar) as: mannose, sucrose, rhamnose, sorbital, melibiose, arabinose and adonitol as a carbon source as well as nitrate and nitrite were not reduced as an electron acceptor, which is similar to **Kim *et al* (2005)**. The present result revealed *E. meningoseptica* was negative for H_2S production. It was unable to reduce sulfur-containing compounds to sulfides during the process of metabolism. It was unable to hydrolyze*o*-nitrophenyl-B-D-galactopyranoside (ONPG) and esculin. Furthermore, *E. meningoseptica* was able to hydrolysis starch, as a source of carbon and energy for growth. An enzyme called alpha-amylase accomplishes use of starch. These findings are in conformity to the previous research performed by **Kim *et al* (2005)**.Both isolates of *E. meningoseptica* were also tested for gelatinase test with isolates from kidney showed positive result. Our findings are in agreement with **Connell *et al* (2011)** who documented *E. meningoseptica* as being able to produce gelatinases, which contributed to their virulence. Nevertheless, the production of certain enzymes promotes the degradation of macromolecules in the medium, there is a clearing in the areas of the medium where bacteria grow **(Pilarski *et al*., 2008)**. According to the results obtained from study by **Mottar *et al* (1989)**, *E. meningoseptica* produces phospholipase C on lecithin agar, enhancing the lipolysis of fat and causing rancidity.

The result of this study demonstrated that *E. meningoseptica* strains were positive for the following conventional tests: production of catalase and oxidase production; growth in 0.5% and 1.5% NaCl nutrient broth; growth at 28˚C and growth on brain–heart infusion agar, trypticase soy agar, blood agar, and nutrient agars. This results is partially agreed with the results of **Ransangan *et al* (2013)** who reported that *E. meningoseptica* strains isolated from American bullfrog (*Ranac atesbeiana*) were Gram staining negative, non-motile, positive for oxidase and catalase, grew at 28˚C and 37˚C but not at 10˚C and 40˚C, respectively. The bacteria were tolerant to NaCl concentrations up to 4% (w/v) but were inhibited at 6% (w/v).In this study both isolates were able to ferment glucose and this result corroborates with the works of **Kim *et al* (2005)**. Additionally, flexirubin-type pigments were not produced from the isolates. The absence of flexirubin pigments is a unique feature differentiating *E.meningoseptica* from all other *Chryseobacterium* species except *C. hominis* **(Vaneechoutte *et al*., 2007)**. Haemolysis is one of the indicators for the virulent of isolates **(Chang *et al*.,2000)**. In this study, isolate from skin exhibited gamma haemolytic activity, and isolate from kidney exhibited beta haemolytic activity. According to **Williams & Lawrence *et al* (2005),** haemolysis is a vital virulence factor for many species of bacteria. Previous research by **Hirono *et al* (1997)** revealed that the haemolytic activity of *Edwardsiella tarda* encoded by gene which are responsible for haemolysis of red blood cells. *E. meningoseptica* was capable of growth at 1.5 % NaCl but unable to grow at 3 %, 6 %, 8 % or 10 % NaCl, indicating that NaCl concentrations above 1.5 % had an inhibitory effect. Previous study by **Bernardet *et al* (2002)** reported that all *Chryseobacterium* species are able to grow on marine agar. Therefore, the salinity range should be determined when NaCl is sufficient for growth. In addition, environmental studies of **Bloch *et al* (1997)** revealed that *Chryseobacterium meningosepticum* can survive in chlorine-treated municipal water supplies, and has become a potential reservoir for infections in the hospital environment.

In this study, API ZYM test detects specific enzymatic activity in bacterial isolates. This method offers a simple way to identify strains based on their enzymatic activities. Therefore, many researchers used API ZYM test to distinguish phenotypic characteristics of bacteria **(Hesami *et al*., 2010; Joh *et al*., 2010; Ugur *et al*.,2012)**.In another study, **Liew *et al* (2012)** enzymatic profiling using the API ZYM system has been useful in the identification of many fermentative and non-fermentative bacteria. Previous study revealed that bacterial enzyme profiles can be used for taxonomy and bacterial typing **(Humble *et al*., 1977)**. Moreover, isolate identifications can be only performed at the genus but not the species level **(Laughon *et al*., 1982).**

The result of this study elucidated that isolates exhibited low level of enzymatic activity, but the reactions were reproducible and reliable for taxonomy identification, in agreement with **Ugur *et al* (2012)**. The API ZYM profiles of kidney isolates were similar to those of skin isolates except for the low level activities of enzymes. Esterase, lipase and leucinearyl amidase detection in skin isolates might be related to the variations in morphology and biochemistry, we hypothesized that the kidney and skin isolates are closely related species of same genus. On the other hand, leucine Arylamidase (LAP) or leucineaminop eptidase is a proteolytic enzyme that catalyzes the hydrolysis of peptides containing leucine.

The kidney isolates were β-hemolytic and might be more virulent than skin isolates. The hemolytic activity of kidney isolates as β-hemolysis may be used as an indicator of enterotoxicity **(Rahim *et al*., 1984).** However, it is not clear to what extent these enzymes are involved in biological events *in vivo* **(Liew *et al*., 2012)**.

Bacterial alkaline phosphatase (APs) is comparatively resistant to inactivation, denaturation, and degradation, and also has a higher rate of activity. Important role in metabolism of different phosphorus containing organic compounds Alkaline phosphatases (APs) occur in a broad diversity of microorganisms and are important in the utilization of phosphoesters, as well as play a role in virulence **(Pandey & Parveen *et al*., 2011)**. Both isolates kidney and skin had higher level activity of enzymeNaphthol-AS-BI phosphohydrolase , which is necessary for bacteria survival. The function of arylamidase enzyme is catalyzing the hydrolysis of N-terminal amino acid from peptides,which contributes to the virulent factor.

The API ZYM test revealed that the kidney and skin isolates shared similar patterns. One distinctive activity was seen in the positive α-glucosidase test, indicating the bacteria's ability to ferment maltose. Kidney and skin isolates were both positive for β-galactosidase and lactose fermentation **Slots *et al*.,1981)**. Esterase activity was seen in all isolates, which indicates that they produced acids and alcohol. Additional β-glucosidase activity proved that the isolates fermented cellobiose **(Hofstad *et al*., 1980).** Both isolates lacked α-mannosidase and α-fucosidase activities **(Laughon *et al*.,1982)**.

In general, there were no obvious correlations between the kidney and the skin isolates. Thus, these isolates were probably different strains, within the same species. Previous studies by **Colding *et al* (1994)** showed that *Chryseobacterium meningosepticum* are divided into subgroups depended on the difference in genetic structure for each other as shows through the difference in pathogenicity. In another study, **Lin *et al* (2004)** indicated that the species *C. meningosepticum*is highly heterogeneous and composed of many subgroups, which may be classified as separate species. This fact confirmed by **Kim *et al* (2005)** separated new genera of *Elizabethkingias pp* from *Chryseobacterium.* In this present study, to determine whether the isolates are closely related, distinguishing their specific enzymatic activity and patterns is necessary. The phenotypic, physiological and biochemical characterizations allow limited discrimination of *Elizabethkingia* species (**Bernardet *et al*., 2005; Hugo *et al*., 2003)**. Further, these methods are useful for preliminary genus identifications **(Bernardet *et al*., 2005; Hugo *et al*., 2003)**. The actual values of these results may be unreliable; therefore, confirmation by another method such as PCR is required **(Hesami *et al*., 2010)**. The BLAST analysis revealed that the 16S rRNA gene sequences of both isolates were more than 97% homology to *Elizabethkingia meningoseptica.*

This result is in agreement with previous study of **Xie *et al* (2009)** performed on frogs with typical clinical signs of cataract disease, identified six *E. meningoseptica* isolates by their 16S rRNA gene sequences and biochemical characteristics. Results of **Kim *et al* (2005)** using 16S rDNA sequences of five strains showing 98.2 to 100 % similarities to *E. meningoseptica.*

CONCLUSION

The results of the present study concluded that *Elizabethkingia meningoseptica* could be detected and identified in fish infected tissue using the universal PCR method accompanied with biochemical and enzymatic profiles. Further work on *E. meningoseptica* pathogenicity on virulent gene expression is under way.

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THE IN VITRO EFFECTS OF SILVER NANOPARTICLES ON BACTERIAL BIOFILMS

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ABSTRACT

Biofilm formation is one of the most important bacterial virulence factors that plays a key role in infections. In the present study, effects of silver nanoparticles were evaluated *in vitro* against bacterial biofilm. Ninety bacterial isolates were selected for study. The Congo Red agar, tube and microtitre assays were used for the detection of biofilm. Antimicrobial effects of silver nanoparticles were determined by the Kirby-Bauer and microdilution methods. The microtitre assay was used to study the biofilm inhibition activity. The most common biofilm producing bacteria was *Staphylococcus aureus.* The power of biofilm production is different among bacteria, and the effect of silver nanoparticles against *Escherichia coli* was less than *Staphylococcus aureus* and *Pseudomonas aeruginosa.* The silver nanoparticles are effective against planctonic and biofilm forms. Because of the importance of biofilm in infectious diseases and the development of drug resistance, silver nanoparticles may be an appropriate way for the control and the prevention of biofilm.

Keywords: Bacteria, Biofilm, Silver nanoparticles

INTRODUCTION

Biofilms are complex and organized communities of bacteria that grow on animate and inanimate surfaces (**Høiby et al., 2014; Southey-Pillig *et al.*,2005**). Biofilm formation is one of the most important bacterial virulence factors that play a key role in serious infections **(Hassan *et al.*, 2011; Joseph, 2003; Odeyemi *et al.*, 2012**). Microbial biofilms can cause skin, wound and teeth infections, are a serious risk factor for patients using artificial biomedical devices such as contact lenses, central venous catheters, urinary catheters, artificial heart valves and intrauterine devices, and can cause serious problems in immune-compromised hosts **(Czaczyk and Myszka, 2007; Mah and O`Toole, 2001)**. It is estimated that about 65% of human infections are related to the biofilm **(Mah and O`Toole, 2001)**.

Considering the importance of biofilm in infectious diseases and increasing drug resistance, scientists are searching for appropriate ways to control and prevent biofilm. In general, therapy with a combination of antibiotics, novel cephalosporin, metals chelating agents, quorum sensing inhibitors, halogens, phage therapy and nanoparticles are used as antibiofilm agents **(Czaczyk and Myszka, 2007**). The diameter of the nanoparticles is about 1 to 100nm and possess sole physicochemical, optical and biological properties (**Whitesides, 2003**). Nanoparticles have wide applications in the medical field, including targeted drug delivery, imaging, artificial implants and are also included in the antimicrobial performance to destroy the wide range of pathogens and drug-resistant organisms (**Samia *et al.*, 2006**). Numerous natural and engineered nanoparticles which have strong antimicrobial properties are silver, gold, magnesium, zinc, copper, aluminum, platinum, palladium, and titanium (**Ravishankar and Jamuna, 2011)**. Silver is a metallic element about the atomic number 47 and silver compounds are used in the treatment of wounds, burns and infectious diseases (**Dunn and Edvards-Jones., 2004**). Silver nanoparticles have been used as a medium to delivery antibiotics and synthetic compounds used in antiseptic filters and coating materials (**Kim *et al.*, 2007**). The objective of our study was to determine *in vitro* effects of silver nanoparticles against bacterial biofilms.

MATERIALS AND METHODS

Identification and biofilm detection

Bacteria were isolated from various samples including ulcer, throat, mucus and urine, and were identified by standard tests (**Mahon, 2014**). Ninety bacterial isolates were selected which included *Staphylococcus aureus* (*S. aureus*) (n=30), *Escherichia coli* (*E.coli*) (n=30) and *Pseudomonas aeruginosa* (*P. aeruginosa*) (n=30). In this study, we used three methods for the detection of biofilm-forming isolates including Congo red agar(CRA), tube method (TM) and microtitre assay (MA) (**Hassan *et al*,2011**).

Congo- Red Agar method

Biofilm production was evaluated using cultivation isolates on Congo Red Agar (CRA), comprising 0.8g of Congo red (Sigma, the USA) and 36g of saccharose (Sigma, the USA) to one litter of brain heart infusion agar (Merck, Germany). Inoculated CRA dishes were incubated at 37°C for 24h followed by storage at room temperature for 48h. The production of rough black colonies by biofilm producing isolates was used to distinguish them from non-biofilm producing strains.

Tube method

Overnight fresh culture of bacterial isolates (equal to 1 McFarland) was prepared; 100µL of this suspension was inoculated into 3mL of Trypticase Soy Broth (Merck, Germany) with 2% glucose (Sigma, the USA) in a glass test tube. After overnight incubation at 37°C, the test tube was decanted and washed 3 times with phosphate buffer saline (pH 7.3), and dried. The adherent biofilm layer was stained with crystal violet 0.1% for 20min, and the excess stain was washed with deionized water. The tubes were dried in an inverted position. The strains which showed an adherent and visible biofilm layer on internal walls of the test tubes were considered as positive.

Synthesis of silver nanoparticles

Chemical reduction method was used for synthesis of silver nanoparticles by Sodium Boron hydride ($NaBH_4$). To stabilize the solution, 0.3% polyvinyl pyrolidine (PVP) was added to the solution to prevent the particles density. The size of nanoparticles in the silver nanoparticle suspension were determined by SALD2101. Suspension of silver nanoparticles became lyophilized powder by freeze-drying method and were kept in a closed container in the refrigerator at 4°C(**Guzmán *et al*., 2009**).

Evaluation of anti-planctonic effects of silver nanoparticles

Antimicrobial effects of silver nanoparticles against the planctonic form were determined using Kirby-Bauer method in Muller-Hinton Agar (Merck, Germany). The lyophilized powder of silver nanoparticles was used for the preparation of discs with concentrations of 5, 25, 100, and 400µg/disc. The petri dishes were evaluated after 24h incubation at 37°C by measuring the inhibition diameter of growth around the disks.

The microdilution assay was also used to determine the MIC of silver nanoparticles against 10 planctonic form of each bacterium(**Guzmán *et al*., 2009**). First, serial dilutions of silver nanoparticles (4.6, 9.3, 18.7, 37.5, 75, 150, and 300µg/ml) were prepared. Then, the wells containing silver nanoparticles and Muller-Hinton Broth (Merck, Germany) were inoculated with the 10^5cfu/ml tested strains and were incubated at 37°C, and growth or no growth was evaluated after 24h. Additionally, the lowest concentration of nanoparticles that allows growth of less than 0.1% of the control culture is considered as minimal bactericidal concentration (MBC).

Evaluation of biofilm inhibition activity of silver nanoparticles

The microtitre assay was used to study the biofilm inhibition activity, and the second concentration of silver nanoparticles was used to obtain the biofilm inhibitory concentration (BIC). After preparing nanoparticles and inoculated 10^5cfu/ml bacteria, the micro-plates were incubated at 37°C. The wells were washed with 200µl phosphate buffer saline two times after 48h. Then, 200µl of 0.1% crystal violet was added to the wells and incubated for 15 minutes; the wells were washed with water and allowed to dry at room temperature. Extra color attached to the surface was removed by ethanol 95%; finally optic density (OD) of stained biofilms was read by ELISA auto-reader at wave length 570nm. The percentage of biofilm inhibition was calculated by the following formula: {The percentage of biofilm inhibition = (OD Control – OD Treat) / OD Control × 100}(**Namasivayam *et al*.,2012**).

Statical analysis

In this study, all tests were repeated 3 times. Data was entered into the SPSS software version 16 and the results were analyzed by One-way ANOVA, LSD post hoc and Two-way correlated analysis of variance tests. In this study, Pv ≤ 0.05 was regarded statistically significant.

RESULTS AND DISCUSSION

Biofilm detection

Biofilms are communities of microbes that its function depends on a complex network of biological interactions (**Li and Tian, 2012**). Microorganisms associated with biofilms behave differently in growth rates. In the present study, three methods were used for biofilm detection, and the MA assay was more sensitive than Congo red agar and tube methods. According to a previous study, the MA is a gold standard assay for biofilm detection (**Mathur *et al*., 2006**). The most common biofilm producing bacteria was *S. aureus* (Chart 1), and biofilm-formation was different among the 3 bacteria (Pv=0.01). The biofilm formation probably is associated with type of clinical samples (Pv=0.05). All *S. aureus* producing biofilm were isolated from wound infections, *P. aeruginosa* producing biofilm were often isolated from urine and throat samples, and *E .coli* producing biofilm were isolated from urine samples.

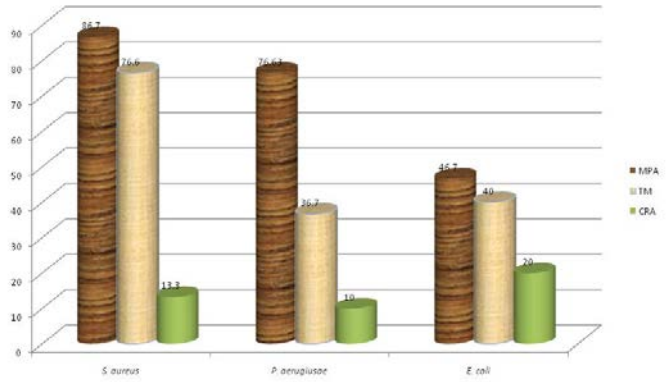

Chart 1 The frequency of biofilm-formation by MA, TM and CRA methods in tested bacteria

Evaluation of anti-planctonic effects of silver nanoparticles

Antimicrobial effects of silver nanoparticles were tested by disk diffusion agar and microdilution methods, and according to the disk diffusion agar results, increasing the concentration of nanoparticles in the discs will be increase the diameter of the inhibition zone (Table 1). Additionally, the post hoc LSD test confirmed this result. Based on the diameter of the growth inhibitory, *P. aeruginosa* isolates showed more sensitivity to nanoparticles in comparison to *S. aureus* isolates and is also more sensitive than *E. coli* isolates.

Table 1 The antimicrobial effects of silver nanoparticles against planctonic form by disk diffusion agar method.

	400µg/disc	100µg/disc	25µg/disc	5µg/disc
S. aureus	14.2*	12.4	6.9	6
E. coli	11.6	9.8	6.5	6
P. aeruginosa	16.6	13.6	10.11	7.9

* The mean growth inhibition size (millimeter) of bacteria with different concentration of silver nanoparticles.

The MIC and MBC results of silver nanoparticles against bacteria are shown in Table 2. One-way ANOVA test showed that the MIC and MBC of nanoparticles were significantly different in various bacteria. The effects of silver nanoparticles against *E. coli* was less than *S. aureus* and *P. aeruginosa*. According to the results of the post hoc LSD test, there are significant differences between isolates of *S. aureus* and *P. aeruginosa* and *E. coli* in terms of MIC and MBC (Pv=0.05), but there were no significant differences between isolates of *S. aureus* and *P. aeruginosa* (Pv=0.741). In this study, based on the results of MIC values, silver nanoparticles had almost the same effect on Gram-positive bacteria (*S. aureus*) and Gram-negative bacteria (*P. aeruginosa*), whilst MIC in 70% of *E. coli* was doubled in comparison to *S. aureus* and *P. aeruginosa*. There are controversy about the effects of silver nanoparticles on Gram-positive bacteria and Gram-negative bacteria. Shrivastava *et al*. (**2007**) reported that silver nanoparticles are generally more active on Gram-negative bacteria than Gram-positive bacteria. The resistance of Gram postive bacteria to silver nanoparticles may be due to the cell wall and the thick peptidoglycan layer of Gram-positive bacteria (**Feng *et al*.**, **2001**). Similar to our data, Doudi *et al*. **(2011)** and Ruparelia *et al*. **(2008)** reported that *E. coli* had a higher resistance to silver nanoparticles than *S. aureus*. Some researcher believe that lipopolysaccharide of Gram-negative bacteria trap positively charged silver nanoparticles and lead to the blocking of nanoparticles. As a result, antibacterial activity of silver nanoparticles needs to reach the cell membrane. In fact, the silver nanoparticles are attached to the surface of cell membranes and can disrupted the performance of the membrane, penetrate the cell and release silver ions.

Table 2 The MIC and MBC results of silver nanoparticles against 10 planctonic form of each bacterium

	Number (%)	MIC (µg/mL)	MBC (µg/mL)
S .aureus	8 (80) 2 (20)	75 37.5	150 75
E. coli	6 (60) 4 (40)	150 75	300 150
P. aeruginosa	9 (90) 1 (10)	75 37.5	150 75

Evaluation of biofilm inhibition activity of silver nanoparticles

The average percentage of biofilm inhibition in *S. aureus*, *P. aeruginosa* and *E. coli* isolates by silver nanoparticles at a second concentrations were 58%, 56% and 44%, respectively. However, the potency of silver nanoparticle biofilm

inhibition was different and the percentage of biofilm inhibition by silver nanoparticles in *E. coli* was less than *P. aeruginosa* and *S. aureus*. Two-way correlated analysis of variance results showed that silver nanoparticles were effective against bacterial biofilm (Pv=0.01). The amount of biofilm inhibition is significantly different between diverse doses of silver nanoparticles. Statistical analysis post hoc LSD tests showed that the percentage of biofilm inhibition at high doses was more than low doses.

In general, the ability of resistance to antimicrobial agents in biofilm is 10 to 1000 times higher than planctonic cells (**Czaczyk and Myszka**, **2007; Taylor and Webster**, **2009**; **Monroe**, **2007**). In this study, silver nanoparticles had potent anti-biofilm effects. Antimicrobial effects of silver nanoparticles have been previously studied (**Taylor and Webster, 2009; Li and Tian, 2012; Velázquez-Velázquez et al., 2015**), but there are a few studies on effects of silver nanoparticles against bacterial biofilm (**Guzmán *et al*., 2009; Mathur *et al*.,2006**). A study from India reported that the production of biofilms in *E. coli*, *S. aureus*, *Salmonella typhi* and *Vibrio cholerae* were inhibited by silver nanoparticles (**Kumar *et al.*, 2012**). Namasivayam *et al.* (**2012**) studied the effects of alone silver nanoparticles and also in combination with several antibiotics, and they concluded that silver nanoparticles made a complete inhibition of biofilm within 24 hours, as well as a good compatibility with combination of silver nanoparticles and antibiotics to inhibit biofilm.

The range of silver nanoparticles size was 50 to 150nm and average particle size was 92nm (Chart 2). The high surface to volume ratio of nanoparticles plays an important role in inhibiting the growth of bacteria. Our study showed that bactericidal effects of nanoparticles is influenced by the particle diameter. Therefore, the choice of synthesis method is effective for controlling the size of silver nanoparticles (**Guzmán *et al*., 2009**). The small particles were more antibacterial and had more antibiofilm activity than large particles, as well as, the triangular-shaped nanoparticles antimicrobial activity was more than spherical particles. In the past studies it was also reported that antimicrobial activity depends on the size of the nanoparticles (**Martinez-Castanon *et al* 2008; Pal *et al*., 2007**).

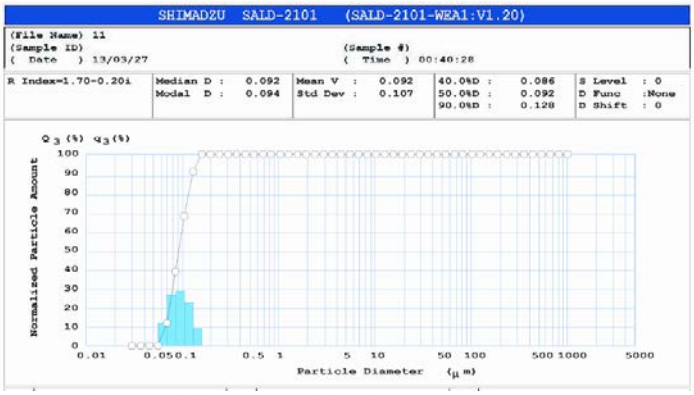

Chart 2 The range of silver nanoparticles size

CONCLUSION

The biofilm formation is associated with type of clinical specimens. The small particles of silver nanoparticles are more antibiofilm activity, and antibacterial activity depends on concentration. This research shows that silver nanoparticles have strong antibacterial and antibiofilm activity. The antibiofilm effectes of silver nanoparticles against bacteria is different and *P. aeruginosa* isolates is more sensitive to nanoparticles. The silver nanoparticles can be used to inhibit bacterial biofilms, and may be useful for treatment of infectious diseases due to biofilm. We recommend conducting more studies concerning this issue and particularly conducting *in vivo* and clinical trial searches before the administration of silver nanoparticles in the treatment of infections due to biofilms.

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BIOLOGICAL ASPECTS OF ANAEROBIC DIGESTION AND ITS KINETICS: AN OVERVIEW

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ABSTRACT

Anaerobic digestion is an anoxic microbiological process occurring in natural environments. Though, the process is considered to be the oldest, its concept relies on the fourth generation biofuel for direct sequestration of carbon-di-oxide into methane. However, the process is successful in natural environments in the efficiency of methane yield with compared to pilot or industrial scale. An overview of an anaerobic digestion and its kinetics is detailed in this review for the successful digestion process, which includes microbiological process, methanogenic interactions, operational conditions of the digester, substrate requirements and its limitation, substrate enhancement by pretreatment and co-digestion process and other rate limiting parameters affecting the process in order to increase the efficiency of methane production.

Keywords: Anaerobic digestion, co-digestion, anoxic, methane, biomethanation, kinetics

INTRODUCTION

The increasing world's population and economical growth in the recent years visualize a dramatic impact over the environment. The augmented anthropogenic activities have developed the crisis in various aspects such as global warming, global energy demand, management of accumulated solid wastes, etc. The anaerobic digestion (AD) process will be a single step solution for this crisis. This process can be achieved in various climatic ranges starting from phsycrophilic to thermophilic. AD is a series of natural process in which the biological waste or organic materials are converted into energy in the form of "Biogas" (a mixture of Methane and CO_2) and the "Digestate" or "Effluent" (N_2 rich product) in the absence of O_2. The production of methane which can serve as the part of energy constraints makes AD process more advantages over aerobic process, which also reduces the excess of sludge production and feasible recovery of useful products; e.g., NH_3 and sulphur (**Bolzonella *et al*., 2005**). This method of digestion uses wide range of bio-materials like livestock manure, agricultural waste, house hold waste, municipal waste water solids, food waste, high strength industrial wastes and residuals, fats, oils and grease (FOG) and convert a mixture of organic substrates into biogas and other valuable products. Biogas can be directly combusted to generate heat for cooking (Domestic use), or to generate electricity, or can be processed into renewable fuel for transportation (http://www.americanbiogascouncil.org, **Department for Environment and Rural Affairs, 2011**). The digestate which is rich in nitrogen source can be directly applied to agricultural fields as biological fertilizer or as soil conditioners. Particularly the liquid and solid materials are highly pliable to this process.

Microbial Archetypes

The archetypal of anaerobic digestion lies vitally within the four stages of biological and chemical process that interpolates Hydrolysis (heterogenous reaction), Acidogenesis, Acetogenesis and Methanogenesis and the conversion is actualized by five groups of microorganisms (Chandrasekar, 2004).

Group 1	-	Hydrolytic Fermentative bacteria
Group 2	-	Obligate hydrogen producing acetogenic bacteria
Group 3	-	Oxidizing acetogenic bacteria
Group 4	-	Carbon dioxide reducing methanogens and
Group 5	-	Aceticlastic methanogens

Hydrolysis and Acidogenesis

The anaerobic digestion process begins with the bacterial hydrolysis of the complex biodegradable polymers (polysaccharides) into soluble form (simple sugars) and make them available for other bacteria (stage 1), which is acquired by group 1 hydrolytic fermentative bacteria. The fermentative hydrolytic bacteria contain the complex consortium of many bacterial species (Bryant 1979). Many of these species are obligate anaerobes and a few facultative anaerobes such as Enteric bacteria, Streptococci were also present (Archer, 1983). Anaerobic bacteria belonging to diverse genera such as *Bacteroides*, *Bifidobacterum, Butyrivibrio, Clostridium, Eubacterium, Ruminococcus* etc., are predominant in anaerobic digester and rumen (Chandrasekar, 2004).

The hydrolysis process is mainly accomplished by extracellular enzymes. The hydrolysis of a complex and insoluble substrates depend on different parameters such as particle size, pH, production, diffusion and adsorption of enzymes to particles. The various hydrolysis rates for different substrates are 0.025-0.200 d^{-1} for carbohydrates, 0.040 - 0.130 d^{-1} for cellulose, 0.005 - 0.010 d^{-1} for lipids (Wilderer *et al*., 2000), food wastes mixtures 0.40 d^{-1} and solid wastes mixtures 0.012 pH-0.042 (Kalyuzhnyi *et al*., 1999). Cellulose is hydrolysed into glucose which is converted into pyruvate by EMP pathway, which is, in turn, fermented into acetate, hydrogen, carbon dioxide or ethanol, lactate, succinate, propionate, and butyrate depending upon low and high partial pressure of hydrogen respectively (Chandrasekar, 2004). Hemicellullose is primarily degraded into pentosans and then to pentoses, mainly cellulose, which is further fermented into lactate, succinate, acetate, propionate, butyrate, hydrogen and carbon-di-oxide. Starch degradation rate is rapid with compared to cellulose and hemicelluloses degradation. It is hydrolysed into glucose which is then fermented (Nagarwala, 1987). Degradation of natural lignin is slow and partial compared with other substrate, but the monomers and oligomers of lignin are comparatively and readily metabolized (Young and Frazer, 1987). Pectins are hydrolysed into pectic acid and methanol, then the pectic acid is initially fermented into galacturonic acid and then into hydrogen and carbon dioxide (Bernhad, 2006).

Hydrolysis of cellulose is carried out by *Clostridium thermocellum, Clostridium stercorarium, Clostridium cellulofementans, Clostridium lentocellum, Clostridium papyrosolvens, Clostridium cellulolyticum, Clostridium joseii, Acetovibrio cellulolyticus, Bacteroides cellulosolvens, Clostridium aldrichii, Clostridium celerecrescens, Clostridium cellulovorans, Ruminococcus flacefaciens, Ruminococcus albus* and *Clostridium popueii* reported by Leschine (1995).

In 1982, Theather & Wood reported the hydrolysis of cellulose by *Bacteroides fibrisolvens, Clostridium cellulobioparum, Ruminococcus flavefaciens, Bacteroides succinogenes, Eubacterium cellulosolvens.* Hydrolysis of suspended

organic solid was reported by *Clostridium thermopalmarium* and *Clostridium novyi* was recorded by Man *et al.* 2010. Hydrolysis is generally the rate limiting step in the overall anaerobic digestion process. It is considered as the first order kinetics (Eqn.1) with respect to the biodegradable solids. The individual rates of the succeeding conversion processes generally fit Monod kinetics as shown in equation 2 (Buswell *et al*., 1995).

$$-dF/dt=K_hF \quad (1)$$

Where, F= concentration of insoluble substrate, $M.L^{-3}$
K_h= first order hydrolysis rate constant, θ^{-1}
T = time, θ^{-1}

$$\mu=\mu_mS/(K_s+S)-b; \quad -dS/dt=X\mu_m/Y \quad (2)$$

Where, μ is the specific growth rate of microorganism, θ^{-1}
μ_m is the maximum specific growth rate of microorganism, θ^{-1}
K_s is the half saturation constant, $M.L^{-3}$
S is the substrate concentration, $M.L^{-3}$
X is the microorganism concentration, $M.L^{-3}$
Y is the growth yield coefficient $M.M^{-1}$
B is the specific decay rate of microorganism, θ^{-1}
θ, M, L^3 is the units of time, mass, and volume respectively.

There are many anaerobic fermentation pathways, but the multifarious group of fermentative anaerobes mostly use lactic acid pathway. Anaerobic bacteria and arachea also use lactic acid pathway and other fermentation pathways including propionic acid fermentation pathway, butyric acid fermentation pathway, stickland fermentation pathway, mixed acid fermentation pathway and solvent fermentation (Stage 2).

The proteins are initially hydrolysed into peptide and then into amino acids by proteolytic anaerobic microorganism. Then these amino acids are decarboxylated and deaminated into organic acids and other products (Ramsay and Pullamanappallil, 2001). The branched amino acids such as valine, leucine and isoleucine are fermented into iso-butyrate, iso-valate and D-2-methyl butyrate respectively. Aromatic amino acids such as phenylalanine, tyrosine and tryptophan are metabolized into phenyl-acetic acid, phenyl-propionic acid, indole, etc., (Elsden *et al.,* 1976).

Lipids are hydrolysed into long chain fatty acids like palmitic acid, stearic acid and glycerol and they are fermented into volatile fatty acids, hydrogen and carbon-di-oxide (Mackie *et al*., 1991). The short chain fatty acids are converted (other than acetate) into acetate, hydrogen and carbon dioxide (Angelidaki *et al*., 2011). Unsaturated fatty acids like oleic, linoleic and linolenic acid are hydrogenated into stearic acid. The products such as organic acids, volatile acids, acetate, long chain fatty acids, alcohol, ammonia, hydrogen, sulphur, formate and other fermentative products like lactate, sucinate, butyrate etc. are formed during fermentative cycle. (Mclnemy and Bryant, 1981).

Acetogenesis

Acetogens are obligatory anaerobic bacteria which uses the reductive acetyl-CoA or Wood-Ljungdahl pathway (Fig.1.0) as their main mechanism for energy conservation and for the synthesis of Acetyl-CoA and cell carbon from CO_2. Acetogens are able to convert H_2 and CO_2 into Acetic acid. They are strict anaerobic bacteria that can grow by the conversion of C_1 compound such as H_2, CO_2, CO and formate into acetate (Diekert and Wohlfahrt 1994). The various substrates such as butyric acid, lactate, acetone, butanol, propionate etc., are converted into acetate, formate, CO_2 and H_2. Acetogens grow on various substrates such as hexoses, C_2 and C_1 compounds. Hexoses are converted exclusively into acetate and therefore, this fermentation is also referred to as homoacetogenesis (Eqn.3) (Volker, 2003).

$$C_6H_{12}O_6 \longrightarrow 3CH_3COOH \quad (3)$$

The acetogenic group further decomposes higher volatile fatty acids into acetate and H_2, which are utilized as substrates for the growth and metabolism of methanogenic bacteria (Ferry and Lessner, 2008). Acetogens also successfully coexist with sulphate reducers and methanogens for various reasons. These include (1) to yield energy from wide and various range of acetogenic reactions, (2) wide range of substrate that enables various types of niche and/or gains energy from a wide range of various substrates, and (3) lesser energetic cost of biosynthesis among acetogens due to the use of reductive acetyl-CoA pathway for both energy production and biosynthesis coupled with the ability to use many organic precursors to produce key intermediate acetyl-CoA (Mark, 2012).

The precursor to the acetic acid is the trimester acetyl-CoA. The key aspect of acetogenic pathway consists of several reactions including the reduction of carbon-di-oxide into carbon monoxide and the attachment of carbon monoxide to a methyl group. The first process is catalyzed by the enzyme carbon monoxide dehydrogenase (Eqn.4). The coupling of methyl group (provided by methylcobalamine) and CO is catalyzed by acetyl-CoA synthetase. In 1936, Wieeringa reported the first acetogenic bacterium *Clostridium aceticum,* and *Moorella thermoacetica.* A clostridium in the Thermoanaerobacteriaceae family has attained wide interest because of its unusual ability to convert glucose almost stoichiometrically into three moles of acetic acid (Eqn.5) (Ragsdale and Pierce, 2008).

$$2CO_2+4H2= CH_3COO^-+H^+ + 2H_2O \quad \triangle G^0 = -95KJ/mol \quad (4)$$

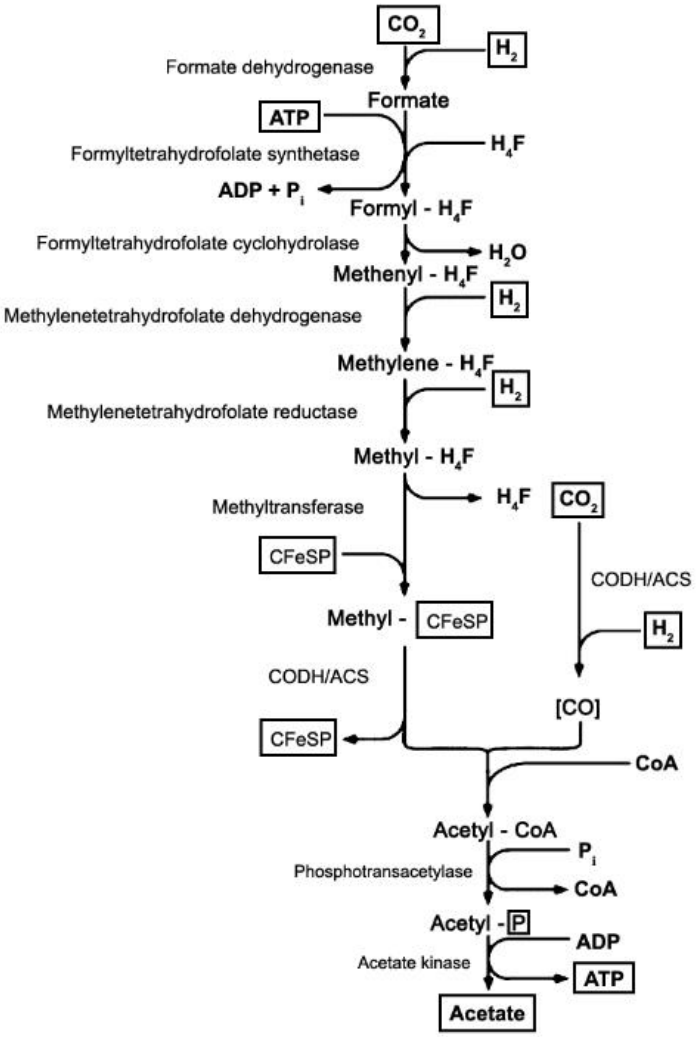

Figure 1 Production of Acetyl CoA and cell carbon from CO_2 by Wood-Ljungdahl pathway (Diekert and Wohlfahrt 1994)

$$C_6H_{12}O_6 \longrightarrow 3CH_3COO^-+3H^+ + \triangle G^0 = -310.9KJ/mol \quad (5)$$

The intermediate metabolic group, the obligate proton – reducing (H_2 forming) acteogenic bacteria (group 2), is a complex species with the following characters: (i) oxidation of alcohols such as ethanol into acetate and hydrogen or complex the corresponding carboxylic acid, (ii) β- oxidation of even-carbon-numbered fatty acids into acetate and odd-numbered fatty acids into acetate, propionate and H2, (iii) decarboxylation of propionate into acetate and CO_2 or acetate and H_2 (Eqn.6) and (iv) other possible reactions (Eqn.7 & 8) (McInerney and Bryant, 1981). Long chained fatty acids are metabolized into acetate by obligate hydrogen producing acetogenic bacteria (group 2). The major products obtained after the digestion of substrate by these two groups are hydrogen, carbon dioxide and acetate (Chandrasekar, 2004).

$$CH_3CH_2COOH + 2H_2O \longrightarrow CH_3COOH + CO_2 + 3H_2 \quad (6)$$
$$CH_3CH_2CH_2COOH + 4H_2O \longrightarrow CH_3COOH + 2CO_2 + 6H_2 \quad (7)$$
$$CH_3CH_2CH_2OH + 3H_2O \longrightarrow CH_3COOH + CO_2 + 5H_2 \quad (8)$$

The interspecies hydrogen transfer between organism producing and consuming hydrogen promotes the decomposition of organic matter in most of the anoxic environments (Fig.2.0) (Walker *et al*., 2009).

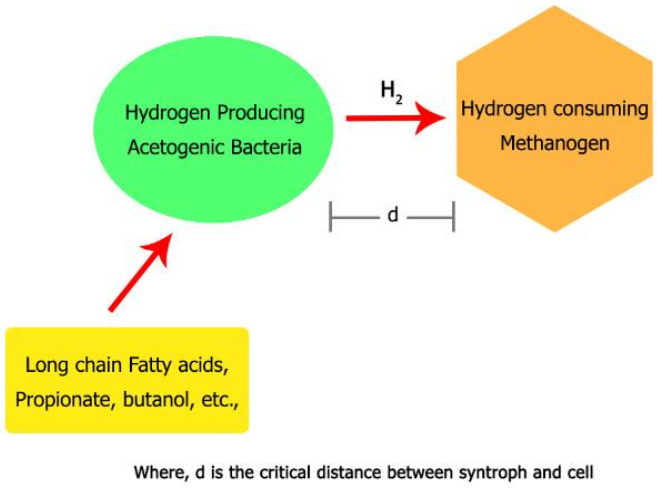

Figure 2 Interspecies hydrogen transfer between the cells (Walket *et al*., 2009).

The process of H_2 transfer between the organisms is referred to as "Syntrophic Acetogenesis" and it is achieved by syntrophic acetogenic bacteria. The syntrophic acetogenic bacteria (in Greek; Syn: together; trophein: eat) putatively grow in a mixed culture with hydrogen consuming bacteria such as methanogens because their metabolism can be inhibited by Hydrogen. The interdependence among these partners may vary from an "Assembly line" – type of cooperation called metabiosis in which only the latter partner in the line profits from the former one but advantages to the former member in the line by the latter partners are negligible. Some examples include the degradation of glucose via acetate into methane by the cooperation of *Acetobacterium woodii* and *Methanosarcina barkeri* (Eqn.9) (Bernhad, 2006).

$$\text{Glucose} \xrightarrow{\textit{Acetobacterium woodi}} 3\text{Acetate} \xrightarrow{\textit{Methanosarcina barkeri}} 3CH_4 + 3CO_2 \quad (9)$$

Syntrophomonas wolfei degrades straight chain fatty acids till octanoate forming acetate and propionate together with either methanogen or sulfate reducers (Schnurer *et al*., 1996). The inter species electron transfer between a propionate oxidizing syntroph, *Pelotomaculum thermopropionicum* SI, and a hydrogen consuming methanogens, *Methanobacter thermoautotrophicus* is facilitated by co aggregation (Ishii *et al*., 2005). Syntrophy model for stimulating coaggregation, the interspecies hydrogen flux between syntrophs and methanogens is estimated on Fick's diffusion law (Eqn.10).

$$J = D_{H2} \frac{C_{H2\text{-}Syntroph} - C_{H2\text{-}\Delta H}}{d} \quad (10)$$

Where, J is the interspecies hydrogen flux
D_{H2} is the H_2 diffusion constant in water (at 55^{O}C)
$C_{H2\text{-}Syntroph}$ is the H_2 concentration immediately outside a syntroph cell
$C_{H2\text{-}\Delta H}$ is the H_2 concentration immediately outside an ΔH cell
d is the average distance between the syntroph and the cells.

The total interspecies hydrogen flux (Q_{H2}) is stoichiometrically correlated with methane mechanism rate and calculated by multiplying J value by the total surface area of hydrogen-releasing syntrophic cell (Eqn.11).

$$Q_{H2} = X_{\text{Symtroph}} . V . A_{Syntroph} . J \quad (11)$$

Where, Q_{H2} is the total interspecies flux
X_{symtroph} is the cell concentration of the syntroph
V is the culture volume
$A_{syntroph}$ is surface area of a syntroph cell.
Q_{H2} between the aggregated and separated cells can be separately estimated (Eqn.12-14) for partially aggregated co-cultures.

$$Q_{H2\text{-agg}} = X_{agg\text{-symtroph}} . V . A_{syntroph} . J . D_{H2} \frac{C_{H2\text{-}Syntroph} - C_{H2\text{-}\Delta H}}{d_{agg}} \quad (12)$$

$$Q_{H2\text{-dis}} = X_{dis\text{-symtroph}} . V . A_{Syntroph} . J . D_{H2} \frac{C_{H2\text{-}Syntroph} - C_{H2\text{-}\Delta H}}{d_{dis}} \quad (13)$$

$$Q_{H2} = Q_{H2\text{-agg}} + Q_{H2\text{-dis}} \quad (14)$$

Where $Q_{H2\text{-agg}}$ & $Q_{H2\text{-dis}}$ are the total interspecies hydrogen flux between the aggregated and separated cells respectively, $X_{agg\text{-symtroph}}$ and $X_{dis\text{-symtroph}}$ are the concentration of aggregated syntroph cells and that of dispersed cells respectively, d_{agg} and d_{dis} are the mean interspecies between the aggregated and dispersed cells respectively (Ishii *et al*., 2005).
Walker *et al*. constructed an archetyphal "community of two" by pairing *Desulfovibrio vulgaris* with a hydrogentrophic methanogens, *Methanococcus maripaldudis* strain S2 in 2009. Syntrophic association between *Clostridium* spp., and hydrogentrophic methanogens, *Methanosarcina* spp. has been investigated by Karakashev *et al*. since 2006. *Methanosarcina barkerii*, growing in a syntrophic co-culture with *Desulfovibrio vulgaris* converts about one-third of the metabolized acetate to carbon dioxide and hydrogen (Schnurer, 1996).
The process in which H_2 and CO_2 convert into acetate by oxidizing acetogenic bacteria (group 3) called homoacetogenesis. Hydrogen and carbon dioxide are converted into acetate by hydrogen oxidizing acetogenic bacteria (Eqn.15&16) (Group 3) (Chandrasekar, 2004) or may be used by the hydrogen utilizing methanogens (Eqn.17) (Gropu 4) for the production of methane or utilized by sulfur reducers and converted into H_2S (Eqn.18&19) (Schink, 1997).

$$4H_2 + 2C \longrightarrow CH_3COO^- + H^+ + 2H_2O \quad (15)$$
$$4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O \quad (16)$$
$$H_2 + SO \longrightarrow H_2S \quad (17)$$
$$4H_2 + SO_4^{2-} + H+ \longrightarrow HS + 4H_2O \quad (18)$$
$$H_2C(NH_3^+)COO^- + H_2 \longrightarrow CH_3COO + NH_4^+ \quad (19)$$

Sporomusa termitida, Desulfomicrobium hypogeum, Acetobacterium psammolithicum Ruminococcus hydrogenotrophicus H_2 utilising acetogenic bacterium have been reported (Bernalier *et al*., 1996). *Eubacterium aggregum,* a homoacetogenic bacteria which was isolated from olive oil mill waste water digestor by Mechichi *et al*. in 1998.

Methanogenesis

Methane production by archaea through biological process is referred to as Methanogenesis (stage 4). The biological methane production Is carried out by a special type of archaea which exhibits a prokaryotic biochemical and morphological features. Studies explored by the Carl Woese say that these organisms are phylogenetically distinct from other prokaryotes and eukaryotes. The existence of methanobacteria is now called methanoarchaea. Archaea is further classified into two main phyla of euarachaeota and crenarachaeota. Methanogens occupy the euarcahaeal branch together with non-methanogenic halophillic, thermoacidophillic and hyperthermophilic archaea. The enzyme system, suggested being an ancestral features of archaea and bacteria, which has subsequently been lost in all but a few lineages of prokaryotes (Juottonen, 2008).
In methanogenesis, about two-thirds of all methane is derived from acetate carboxylation whereas about one-third is from CO_2 reduction with small amounts coming from other substrates (Zinder, 1990). The biomethanation occurs through various processes as follows; reduction o f CO_2 (Eqn.20) including the reduction of carbon-di-oxide into the formyl level (Eqn.21), reduction of the formyl group into formaldehyde level (Eqn.22&23), reduction of the methylene group into methyl level (Eqn.24), conversion of the methyl group into methane via transfer of the methyl group to Co-enzyme M (Eqn.25); reductive demethylation CH3-S-CoM into methane (Eqn.26 & 27), electron transport and bioenergetics through electron carriers, hydrogenase, formate dehydrogenase, alcohol dehydrogenase; conversion of acetate into CO_2 and CH_4 (Eqn.28 & 29) via the activation of acetate into acetyl Co-A, decarbonylation of acetyl Co-A, methyl trtansfer to HS-CoM, reductive demethylation of CH_3-S-CoM into methane, electron transport and bioenergetics and other enzyme activities; disproportionate of methanol or methylamines to methane and carbon dioxide (Eqn.30-32); methyl transfer reaction leading to methane, oxidation of the methyl group into CO_2 and electron transport and bioenergetics by methanol oxidation to CO_2, methanol reduction to CH_4 (Ferry, 1992).

$$4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O \quad \Delta G^{O'} = -130.4 \text{ kJ/ mol} \quad (20)$$

$$CO_2 + MF + H_2 \longrightarrow \text{Formyl-MF} + H_2O \quad \Delta G^{O'} = +16 \text{ kJ/ mol} \quad (21)$$

$$\text{Formyl-MF} + H_4MPT \longrightarrow \text{5-formyl-}H_4MPT + MF \quad \Delta G^{O'} = -4.4 \text{ kJ/ mol} \quad (22)$$

$$\text{5-formyl-}H_4MPT + H^+ \longrightarrow \text{5,10 - methenyl-H4MPT}^+ + H_2O \quad \Delta G^{O'} = -4.6 \text{ kJ/ mol} \quad (23)$$

$$\text{2,10-methylene-}H_4MPT + F_{420}H_2 \longrightarrow \text{5 methyl-}H_4MPT + F_{420} \quad \Delta G^{O'} = -5.2 \text{ kJ/ mol} \quad (24)$$

5 methyl-H_4MPT + F_{420} + HS-CoM ⟶ CH_3-S-CoM + H_4MPT
$\Delta G^{O'}$ = -29.7 kJ/ mol (25)

CH_3-S-CoM + HS-HTP ⟶ CH_4+CoM-S-S-HTP
$\Delta G^{O'}$ = -45 kJ/ mol (26)

CoM-S-S-HTP + H_2 ⟶ HS-CoM + HS-HTP
$\Delta G^{O'}$ = -40 kJ/ mol (27)

$CH_3COO^+ + H^+$ ⟶ CH_4 + CO2
$\Delta G^{O'}$ = -36 kJ/ mol (28)

CH_3COO^- + Co-A + ATP ⟶ $CH_3CoSCoA$ + Pi (29)

$CH_3OH + H_2O$ ⟶ $CO_2 + 6e^- + 6H^+$ (30)

$3CH_3OH + 6e^- + 6H^+$ ⟶ $3CH_4 + 3H_2O$ (31)

$4CH_3OH$ ⟶ $3CH_4 + CO_2 + H_2O$
$\Delta G^{O'}$ = -103 kJ/ CH_4 (32)

These conversions are attained by two groups of methanoarachaea such as carbon-di-oxide reducing methanogens (Group 4) and acetate utilizing methanogens or acetolastic methanogens (Group 5). The methane produced in the anaerobic reactor can be calculated using mass - calculations. Calculation for the conversion of mass based data into volumetric basis requires only a measurement or estimate the wet density (wet mass volume^{-1}) (Richards *et al*., 1991). Biogas produced in anaerobic digestion consists of methane, carbon dioxide, water vapor and other gases in trace amounts. The calculation for the standard biogas volumes are represented in equation 33.

$$V_O = DBF_t \text{ X } V_t \quad (33)$$

Where, V_O is the dry (non water) biogas volume at 0°C
DBF_t is the dry(non-water) biogas volume at 0° C
V_t biogas volume measured at temperature T.

Richards *et al*. (1991) has performed a temperature versus dry biogas factor regression (Eqn.34) to simplify this application. The linear regression (R = 0.9997) covers a range of common ambient temperatures (15° – 27°C). The mass loss due to evaporation is calculated from the biogas volume (Eqn.35). The temperature/vapor density relationship is exponential (Eqn.36) and an exponential regression (Eqn.37) is performed. Then the corrected mass loss is represented in the equation 37. Mass removal rate ($MRR_{m,}$ g(Kg.d)$^{-1}$) is defined as MR_m divided by the net reactor mass (Kg) and length of the time interval (day).

$$DBF_t = 1 - 0.0045 \text{ x T} \quad (34)$$

Where, T = biogas temperature (°C) within the range 15°C to27°C

$$W = V_t \text{ x } D_W \quad (35)$$

Where, W is the mass of water lost by evaporation in biogas (g water (biogas)$^{-1}$)
V_t is the biogas volume at ambient temperature
D_w is water vapor density (g water)

$$D_w = 0.005396e^{(0.05808T)} \quad (36)$$

Where, D_w is the water vapor density
T is the ambient biogas temperature (15°C – 27°C)

$$MR_m = I - F - W \quad (37)$$

Where, MR_m is mass removed (or) during interval (mass loss method)
I is initial reactor mass (beginning of interval)
F is final reactor mass (end of interval)
W is mass of water lost by evaporation in biogas.

The biogas based method of determining removals calculates the mass of dry biogas produced. The biogas mass is calculated using the molecular weight of methane and carbon-di-oxide respectively (16 and 44 g mol^{-1},) and the molar volume of an ideal gas at STP (22.41310 mol^{-1}) (Eqn.38) and by substituting CO_2 = 100 – CH4 in equation 38 and simplifying the constant results (Eqn.39) and obtained the mass removal rate MRR_b, (g (Kg.d)$^{-1}$) mass (Kg) and the time interval (day). These two independent variables (Eqn.37 & 39) yields the equal results ($MRR_m = MRR_b$).

$$B = V_o \text{ x } \frac{(16 \text{ x } CH_4 /100) + (44 \text{ x } CO_2 /100)}{22.413} \quad (38)$$

Where, B is biogas mass (g)
V_o is dry biogas volume at STP
CH_4 biogas normalized methane content (volume percent)
CO_2 biogas normalized carbon dioxide content (volume percent)

$$MR_b = B = V_o [1.963 – (0.01249 \text{ x } CH_4)] \quad (39)$$

Where, MR_b is mass removed (g) (biogas method)
Vo is dry biogas volume STP
CH_4 is biogas normalized methane content (volume percent).

Determination of hydrolytic water consumption includes both the converted substrate mass and water consumed during hydrolysis process for methane fermentation. For example, starch or cellulose with n hexose units shows that 10% of the mass of biogas produced originates as water (Eqn.40). The hydrolytic factor H can be determined as shown in equation 41, which assumes that the difference between the steady state volatile solids (VS) removals and mass removals is hydrolytically-consumed.

$(C_6H_{10}O_5)n + nH_2O \rightarrow 3nCO_2 + 3nCH_4$
Molecular weight
$162n + 18n \rightarrow 180n$
Substrate mass converted + water mass consumed → biogas mass produced (40)

$$H = 1 - (VS\ RR/MRR) \quad (41)$$

Where, H is hydrolysis factor, g water consumed (g mass removed)$^{-1}$
$VS\ RR$ is VS removal, g VS (Kg.d)$^{-1}$
MRR is mass removal rate (mass loss and/or biogas basis), g (Kg.d)$^{-1}$

The second method for the determination of H is based on the fermentation stoichiometry equation (Eqn.41), in which it is assumed that ammonia released can be retained in the solution and balanced by bicarbonate derived from carbon-di-oxide (Buswell equation). The coefficient generated by the equation (Eqn.41) is used to calculate the H factor (Eqn.42). For example, c moles of N, x moles of H_2O are consumed; y moles of CH_4 and z moles of CO_2 are produced (Buswell *et al*., 1959; Richards *et al*., 1991). The equation can be simplified to calculate H factor (Eqn.43) by substituting the equation 42.

$$C_n H_a O_b N_c + [n - 0.25a - 0.50b + 1.75c] H_2O \rightarrow [0.50n + 0.125a - 0.25b + 0.375c] CH_4 + [0.50n - 0.125a + 0.25b - 0.625c] CO_2 + cNH_4^+ + cHCO_3^- \quad (41)$$

$$H = (18 (x\text{-}c) - c) / (16y + 44z) \quad (42)$$

$$H = (18n - 4.5a - 9b + 12.5c) / (30n - 3.5a + 7b - 33.5c) \quad (43)$$

The calculation based on Hydraulic Retention Time (CSTR) assumes the use of Q as the through put, the rate of mass inflow equals the rate of mass outflow. This is technically incorrect as the mass removed as biogas is ignored. The HRT is conventionally defined as the reactor volume (V) divided by the volume of liquid throughput (Q, volume time^{-1}). The Mass Removal Rate (MRR) can be determined by the difference between the rate of inflow (Qo) and the rate of effluent outflow (Qe). The determination of actual hydraulic retention time is represented in the equation 44.

$$HRT_{actual} = M/Qe = MX/QeX = SRT \quad (44)$$

here, HRT_{actual} is the Hydraulic retention time
M/Qe is the actual residence time of liquids in reactor (by outflow)
M is the net mass loaded at rate Qo (mass time^{-1})
X is the microbial Vs concentration in reactor and effluent.

On the contrary, the retention time based on the inflow (M/Qo is actualized as HRT_i) is not the actual retention time, but it is still needed as Qo is an independent control variable. In the CSTR system Qe can be significantly less than Qe as represented in equation 45, resulting in retention times that are much longer than the HRT_i. But the ratios Qo/Qe and SRT/HRTi are found to be increasing with the extent of substrate conversion. SRT/HRT_i ratios approximately to 2 are possible in high solids reactor fed with highly biodegradable substrate.

$$SRT = M/Qe > M/Qo = HRT_i \quad (45)$$

The first order CSTR kinetic reaction (Eqn.46) is modified by Richards *et al*. (1991) on the mass basis, accounts for biogas mass losses with distinguishing Qo and Qe (Eqn.47).

Change Substrate Mass = Influent Substrate Mass – Effluent Substrate Mass – Substrate Mass Removed in Biogas (46)

$$M\ dS/dt = QoSo - QeSe - kMSe \quad (47)$$

Where, M is reactor wet mass

d*S*/d*t* is rate of change of substrate concentration, mass mass^{-1}
*Q*o is rate of inflow, wet mass time^{-1}
*Q*e is rate of outflow, wet mass time^{-1}
*S*o is influent substrate concentration, substrate mass (wet mass^{-1})
K is first order rate coefficient, time^{-1}

By definition, d*S*/t at steady state equals to zero (Eqn.48), thus

$$QoSo - QeSe - kMSe = 0 \quad (48)$$

A constant reactor mass requires that inflow equal outflow plus biogas mass removals (the removal rate (*kMS*e) divided by the (1-H) terms yields the mass removal rate) (Eqn.49).

$$Qo = Qe + kMSe /(1-H) \quad (49)$$

Where, *H* is substrate hydrolysis factor, more water consumed (mass loss)$^{-1}$

Substituting the relationship for *Q*o and the definition of *SRT* (*M*/*Q*e) in equation 48 and solving k are shown in equation 50 & 51 on the basis of HRT_i. Substrate concentrations *S*o and *S*e are easily calculated from biodegradable *VS* (*BVS*) loading and removal rates (Eqn.52&53) (Richards *et al*., 1991).

$$K = (So\text{-}Se)/[Se\ HRT_i(1\text{-}So/(1\text{-}H)] \quad (50)$$
$$K = (So\text{-}Se)/[SeHRT_i(1\text{-}Se/(1\text{-}H)] \quad (51)$$
$$So = BLR/(1000/HRT_i) \quad (52)$$
$$Se = (BLR\ \text{-}RR)/[(1000/HRT_i) - MRR] \quad (53)$$

Where, *BLR* is *BVS* loading rate, g *BVS* (Kg.d)$^{-1}$
RR is *VS* removal rate, g *VS* (Kg.d)$^{-1}$
MRR is mass removal rate, g (Kg.d)$^{-1}$

The relationship between the biogas production and the substrate utilization can be estimated by this kinetics reaction.

Sulfate Reduction in Anaerobic Digestion

The sulfate reducing bacteria are normally dominant in various natural habitats such as fresh and marine sediments and also in anaerobic digester. In anaerobic digestion, the hydrolysed products such as monosaccharide, amino acids, higher fatty acids and alcohols and the intermediate products like acetate, H_2 and CO_2 (Eqn.54-59) with the presence of sulfate and sulfite are used by the sulfate reducers, which use these compounds as electron acceptor during the oxidation of organic materials under anaerobic conditions. This condition creates a critical competition in substrate utilization used in sulfite reduction instead of fermentation. If Acetogenesis and Methanogenesis are reduced, this apparently reduces methane formation. By these, the sulfate reducing bacteria can utilize 53-93% of the available substrate electrons in anaerobic digestion as reported by Zaid *et al*. 1986.

$$4H_2 + SO_4^{2-} + H^+ \longrightarrow HS^- + 4H_2O$$
$$\Delta G^{O'} = -151.9\ kJ/\ mol \quad (54)$$

$$Acetate + SO_4^{2-} \longrightarrow 2HCO_3^- + HS$$
$$\Delta G^{O'} = -47.6\ kJ/\ mol \quad (55)$$

$$Propionate + 3/4SO_4^{2-} \longrightarrow Acetate\text{-} + HCO_3^- + 3/4HS^-$$
$$\Delta G^{O'} = -37.7\ kJ/\ mol \quad (56)$$

$$Butyrate^- + 1/2SO_4^{2-} \longrightarrow 2Acetate\text{-} + 1/2HS^-$$
$$\Delta G^{O'} = -\ 27.8 kJ/\ mol \quad (57)$$

$$Lactate\text{-} + ½\ SO42\text{-} \longrightarrow Acetate^- + HCO3^- + 1/2HS^-$$
$$\Delta G^{O'} = -80.0\ kJ/\ mol \quad (58)$$

$$Ethanol + 1/2SO_4^{2-} \longrightarrow Acetate^- + 1/2HS^- + ½\ H^+ + H_2O$$
$$\Delta G^{O'} = -66.4\ kJ/\ mol \quad (60)$$

Substrate for Anaerobic Digestion

The successful anaerobic digestion process can be achieved using suitable substrate by utilizing various organic materials to produce energy.

Municipal Solid Waste and Industrial wastes

The solid wastes are produced by day to day human activity and abounded by the people. These domestic wastes mainly consist of biodegradable waste including food waste, the remainings vegetable and fruits, green waste, cellulosic materials like papers, carton etc.

The wastes produced by household, market, hotel, sewage waste and sludge, human excretory waste can act as a good source for anaerobic digestion. Various types of municipal solid waste are exploited by various researchers for successful digestion process that encompasses food market waste, utilization of fruit and vegetable waste, canteen wastes, market waste, food waste (Forster *et al.,* 2008). Rao and Singh (2003) used various wastes such as food wastes from fruits and vegetable markets, household wastes and wastes from hotels and juice centers (Dawei *et al.,* 2006).
Deploying of domestic kitchen wastes is useful for biomethanation process (Chandrasekar, 2004) and with black water (Elimitwalli *et al*., 2006). Consumption of olive oil mill wastes include house hold wastes, sewage slaughter house waste water and sewage water, organic fraction of municipal solid waste, waste activated sludge and sewage sludge (Forster *et al*., 2008; Yebo Li *et al*., 2011). Yen and Brune (2007) employed algal sludge and paper waste for biogas production.

Agricultural Waste

The agricultural wastes starting from agricultural bi-products to farmyard dung can act as a good basis of feedstock for anaerobic digestion process. They include waste from livestock, cow slurry, pig slurry, chicken slurry, farmyard manure, harvest remains and garden wastes, energy crops, feedlot runoff, silage juices, waste and waste water from agricultural related process (Wilson, 2004). The cattle dung not only acts as a good source of feedstock but also found to be the basis of inoculum for anaerobic digestion for the production of methane and other bi-products. The farmyard materials, agricultural residues and cattle litters possess rich source of carbohydrates proteins and other growth nutrients required for digestion process. Holm-Nielsen *et al*. 2009 employed animal manure, animal waste and slurries for anaerobic process. Deploying of piggery wastes, agricultural wastes, lignocellulosic biomass, energy crops like aquatic and marine plants and grasses and woods is helpful for the production of methane gas via anaerobic digestion (Alastair *et al.,* 2008).
Prochnow *et al*. 2009 employed different variety of grass species for biogas production via anaerobic digestion. Leaf residues of *Gulmohar*, *Leucacena leucocephala*, *Acacia auriculiformis*, *Dalbergia sisoo* and *Eucalyptus tereticonius* and plant residues like Lantan, wheat straw, apple leaf litter and peach leaf litter supplemented with cattle dung are used for biogas production process (Yadavika *et al*., 2004). Deploying palm oil mill waste with co-digestion of cattle manure accompanied with agro wastes and energy crops is used for the fermentation process (Cairnats *et al*., 2010). Grass silage and animal manure, lignocellulosic crop residues are used in co-digestion process for the production of methane (Yebo Li *et al*., 2011). Therefore any combination of solid waste can be incorporated for successful anaerobic fermentation depending upon its regional availability.

Effect of Various Parameters in Anaerobic Digestion and Methane Production

Substrate Enhancement by Pretreatment and Co – Digestion

Naturally hemicellulosic and lignocellulosic materials resist biological degradation highly as the anaerobic digestion process is highly operated with substrate rich in cellulosic materials (Taherzadeh and Karimi 2008). The digestion process initiated by hydrolysis continues to methanogenesis and ends with methane as the end product. The efficient methane production is highly dependent on hydrolytic process. In order to obtain the effective hydrolysis process, the lignocellulosic compounds are subjected to different pretreatment methods (Venkata *et al*., 2008). The pretreatment process can be achieved by various methods including physical, chemical, physhicochemical and biological methods for the better production of ethanol and biogas. Physical pretreatment methods like milling, ultrasonificaton, microwave treatment, irradiation, thermal treatment and other pressurized methods are adopted by various researchers (Elliott and Mahmood 2007; Yu *et al*., 2010).
Chemical and physicochemical method comprises ammonia explosion method, thermal explosion method, thermo-chemical methods, alkali treatment, acid treatment, gas treatment, treating with ozone, treating with oxidizing agent and other solvent extraction methods for methane and ethanol production (Elliott and Mahmood 2007; Xialing *et al*., 2008). Biological pretreatment encloses treatment with the fungus and actinomycetes for depolymerization and partial hydrolysis of hemicelluloses (Venkata *et al*., 2008).
Secondly, lipids and proteins represent an important fraction of the particular organic materials. In the anaerobic treatment process, the methane production is slowed down or impaired by high suspended solids particularly by lipids and fats (Saxena *et al*., 1986). In anaerobic digestion process, pretreatment of fat is achieved by enzymatic methods, alkaline hydrolysis method and by other methods (Masse *et al*., 2003). The anaerobic digestion can be further enhanced by co-digestion process. The co-digestion process is the combination of one or more typical substrate utilized for the operation of anaerobic digestion and various co-digestion processes are successfully adopted (Maritza *et al*., 2008).

Effect of Ammonia

Various substrates are utilized for the anaerobic digester, which contains carbohydrates, proteins, lipids and other nutrient required for the development for microbial consortium for methane production. When the protein and urea rich substrates are hydrolysed, it results in the generation of free ammonia from the substrate (Kayhanian, 1999). The free ammonia has been suggested to be inhibiting digestion process since its membrane is permeable. The amount of ammonia produced during the degradation of biological compounds in an anaerobic digestion is estimated by the following stoichiometric equation (Eqn.61) (Tchobanoglous *et al*., 1993).

$$C_a H_b O_c N_d + \frac{4a-b-2c-3d}{4} H2O > \frac{4a+b-2c-3d}{8} CH4 + \frac{4a-b+2c+3d}{8} CO_2 + dNH_3 \quad (61)$$

Nitrogen is an essential nutrient for the growth and development of microorganism, which are available in the form of NH_3. Ammonia can inhibit the cellular metabolism by various mechanisms like altering the intracellular pH, increasing the energy requirement for cellular maintenance, blocking or inhibiting the specific enzymatic reactions (Elliott and Mahmood., 2007). These free ammonia are hydrophobic, membrane permeable in nature, easily diffuse passively into the cells causing proton imbalance and potassium deficiency. When its concentration is below 200mg.L^{-1}, it supports the growth of anaerobic microorganisms. The concentration of ammonia increases in the range of 4051-5374mg.L^{-1} and hardly affects acidogenic population and methanogenic activity up to 56.5% (Liu and Sung, 2002). In order to overcome this, ammonia present in the substrate is removed by physical and chemical method. Both air stripping and chemical precipitation method in waste water matrix have been proven to be technically feasible (Kabdasli *et al*., 2000). The microorganisms immobilized on various inert materials like activated carbon, clay and zeolites have been established to increase methane production and make the process more stable. Various ion changers and adsorbants made up of natural zeolite and glauconite were used to reduce ammonia inhibition in digestor process (Borja *et al*., 1996 and Hansen *et al*., 1998).

EFFECT OF LIPIDS, VFA'S AND pH

The lipids and fats are added to the reactor system in order to enhance the production of methane (Ahring, 2003). The reactor system is operated by lipid rich wastes obtained from various point sources such as slaughter house, food processing industries, dairy industries, edible oil processing industries, olive oil processing industries, fish processing industries etc. The lipids and fats directly affect the reactor operation by clogging and floating of biomass due to the adhesion of fat during the operation conditions and it will lead to low efficiency of the system (Pereira *et al*., 2004). In the digestion process the lipids are primarily hydrolysed into glycerol and they free fatty acids. Further, glycerol is converted into acetate by acidogenesis and long chain fatty acids. The long chain fatty acids are converted into acetate or propionate or hydrogen through β – oxidation pathway (syntrophic acetogenesis). The LCFAs are suggested to be the actual toxic agents, which in case of affecting the growth and activity of the acetolastic and hydrogenotrophic methanogens leads to permanent failure of the system. The increased VFAs and pH in the digestion process leads to "reactor upset" and reduces methane yield (Veeken and Hamelers., 2000). However, anaerobic reactor can be successfully operated at wide pH range depending on the methanogenic diversity. The total ammonia and nitrogen, pH and volatile fatty acid adversely modulate the microbial growth and methane yield (Hansen *et al*., 1999). The pH fluctuations in the reactors occur due to various conditions like free ammonia concentration, VFAs productions, substrate characteristics. But the major cause for pH alterations is due to the accumulation of free ammonia and it has been suggested to be the actual toxic substance (Borja *et al*., 1996). The accumulation of free ammonia in digester system leads to the increased production of VFAs. In case it again reduces the pH of the system, instability will occur in the process (Angelidaki and Ahring, 1992). But various studies show that the reactors operate at various pH ranges depending upon the product consideration and substrate characterization (Yuan *et al*., 2006). The methanogenic archaea mostly prefers neutral pH to the metabolism of acetate, H_2 and CO_2 (Taconi *et al*., 2008). The optimum pH range relies between6.5–8.5 and the methanogenesis process is completely inhibited pH below 5.0 for the enhanced methane production (Kim *et al*., 2003). In order to overcome this, proper pretreatment process should be adopted depending upon the strength and type of the substrate used. Further, the substrate type and its particulate concentration confined microbial consortium should be selected to obtain the enhanced methane yield.

EFFECT OF TEMPERATURE

Anaerobic digestion is operated at several temperature ranges for methane production process including thermophilic (45°C and above), mesophilic (25-45°C), psychrophilic (10-15°C) have been reported by Stanier and Niel, 1962. But, mostly the anaerobic digestion process is carried out at mesophilic to thermophilic conditions. Nowadays it is possible to operate digestion process under psychrophilic condition for methane production (Katarzyna *et al*., 2013). However, the operational condition of digestion process under different temperature has its own advantages and disadvantages. The mesophilic digestion process is more stable and easy to control thermophilic conditions. When thermophilic process is compared with mesophilic process, the rate of digestion will be greater than that of one-third of mesophilic process which results in high yield of methane (Chandrasekar *et al*., 2004). The Psychrophilic Anaerobic Digestion Process (PADP) or Low Temperature Digestion Process (LTDP) can reduce the operational cost. Further, it can improve the energy balance of the plant (McKeown *et al*., 2012). But more or less thermophilic process is found to be more successful in practical because it not only increases the methane yield but also lowers the retention time, improves dewaterability of the sludge, increases the destruction of pathogens and increases hydrogen yield which are further utilized by hydrogenotrophic methanogens for the enhanced methane production (Vindis *et al*., 2009). Various operational temperatures, which indicates that organic loading rate and microbial diversity particularly methanogenic consortium plays a vital role for methane formation. In the northern part of India, it is recorded that a short fall in biogas output during the winter and in some part of country the digestor performance has also affected higher temperature (Ramasamy, 1997). But the diurnal temperature does not have any negative impact on the digestion process. The above mentioned conditions and the fluxes in methane production are mainly due to the inadaptability of microbial diversity present in the digestor which is operated in continuous culture system.

EFFECT OF METAL IONS

It is a biological process in which metal ions are produced from organic load used in the digestion process itself. It consists of light metal ions and heavy metal ion (sewage, sludge and other waste water) or it contains additional pH adjustment chemicals depending upon the varieties of substrate (Graddy *et al*., 1999). Most of the light metal ions are required for microbial growth which includes sodium (Na), Potasium (K), Magnesium (Mg), Calcium (Ca) and Aluminium (Al) at specific rate. If the salt concentration increases, it will lead to the osmotic imbalance which slows down the microbial growth but it will result in the death of cell in the excess rate (Soto *et al*., 1993). The heavy metals are present in the sewage, waste water and sludge etc. at a significance concentration depending upon their process of production. The heavy metal ions like Chromium (Cr), Iron (Fe), Cobalt (Co), Copper (Cu), Zinc (Zn), Cadmium (Cd) and Nickel were reported by Jin *et al*. 1998. Not like other compounds these are non biodegradable and accumulate in digestor which leads to process imbalance by toxic effects (Steritt and Lester, 1980). Numerous anaerobic reactions take place in the presence of heavy metal ions as a part of their essential enzymes. Heavy metal concentration present in ten methanogenic isolates are found in the following order: Fe>>Zn>/ Ni>Co=Mo>Cu (Takashima and Speece, 1989). The increased heavy metal ion concentration in anaerobic digestion process causes change in enzyme function, alters structure by binding with thiol, binding to protein molecules and it replaces natural occurring metal ions present in the prosthetic group (Vallee and Vulner, 1972). The stimulatory and inhibitory effects of these metal ions mainly depend on the chemical forms of the metal process related by pH or Redox potentials and the total metal ion concentrations (Zayed and Winter 2000). These metal ions not only inhibit the process by individual concentration but also play a synergistic and/or antagonistic effect by the concentration of two or more metal ions. The light metal ion combination of potassium K and Ca significantly increase the antagonistic nature that of K alone (Kugelman and McCarty, 1964). The antagonistic and or synergistic effects of the mixed heavy metal ions such as Cr-Cd, Cr-Pb, Cr-Cd-Pb and Zn-Ca-Ni are reported by Lin, 1992.

CONCLUSION

Anaerobic digestion process occurs naturally and it is a key point for solid waste and waste water management. The byproduct produced at the end of digestion process includes methane, hydrogen, and digestate (Fertilizers) with potential commercial value. This process is also environmental friendly (Eco-Neutral) and will be an effective remedy for carbon-di-oxide sequestration. However, the process is operated under various parameters which directly or indirectly depend on the growth of microbial consortium. Starting from acidogenesis (stage1) to methanogenesis (stage4), the process is interdependent with each other. If anyone of the stage fails to function, it will automatically reduce the efficiency of the digestion process. The efficiency of the digestion process mainly depends upon the type of substrate, microbial consortium, and operational conditions. Obtaining the information on anaerobic digestion process is necessary for the successful implementation of the process. So, it is suggested that every small things should

necessarily be considered for the sophistication of microbial consortium for enhanced digestion process.

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