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ARTICLES

African Journal of Microbiology Research

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Full Length Research Paper

Antibacterial and antioxidant activities of bilberry (*Vaccinium myrtillus* L.) *in vitro*

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Antibacterial and antioxidant activity, total phenolic and flavonoid concentrations of water, ethanol and ethyl acetate extract of fruits and leaves of *Vaccinium myrtillus* L. were studied. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) have been determined. Testing was performed on 30 clinical isolates, including strains of *Escherichia coli*, *Enterococcus faecalis* and *Proteus vulgaris*. The values for MIC were in the range from 5 to 40 mg/ml. The most sensitive bacterial strain was *Enterococcus faecalis* MF-Ef8 strain. The ethanol extract of fruits of *V. myrtillus* was found the most active. The total phenolic content was determined using Folin-Ciocalteu reagent and ranged between 31.44 to 119.17 mg GAE/g. The concentration of flavonoids in extracts was determined and the highest amount was in ethyl acetate extract of leaves of *V. myrtillus*. Antioxidant activity was monitored spectrophotometrically using 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent. The highest capacity to neutralize DPPH radicals (94%RSA) was found in the ethanol extract from fruits and in the water extract from leaves of *V. myrtillus*. The results of the total phenolic content determination of the examined extracts indicate that bilberry extracts are a rich source of phenolic compounds and also possess a significant antioxidant activity and moderate antibacterial activity.

Key words: Plant extracts, phenols, flavonoids, *Escherichia coli*, *Enterococcus faecalis*, *Proteus vulgaris*.

INTRODUCTION

Bilberry (*Vaccinium myrtillus* L.) is a deciduous shrub growing to 50 cm, with elliptical leaves. The flowers are single on short stems. The fruits are berries, globular, dark purple, juicy and sour (Kovačević, 2002). In many European countries, the bilberry is one of the most economically important wild berry species (Tomićević et al., 2011).

Different *Vaccinium* species (*V. myrtillus*, *V. vitis-idaea*, *V. macrocarpon*) are used in phytomedicine and pharmacy. Fruits of these species may have additional health benefits as they are rich in phytochemicals such as anthocyanins which are responsible for their red, purple and blue colours. Previous studies demonstrated that plants with high content of anthocyanins, had

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significant antibacterial effect (Hearst et al., 2010). *In vitro* studies indicate that anthocyanins and other polyphenols in berries could be substantial in the treatment of heart disease (Basu et al., 2010; Routray and Orsat, 2011), including antioxidant (Denev et al., 2010) and antiadhesion activity against bacteria (Huttunen et al., 2011). Compounds such as quercetin and phenolic acids could play an important role in the possible health effects of berries (Paredes-López et al., 2010). Bilberry fruits contain up to 10% tannins, anthocyanins, organic acids, and pectins. The leaves contain tannins, flavonoids, and a small amount of arbutin. In traditional medicine, fruits of *V. myrtillus* are much used as antidiarreal while leaves are used as astringent and diuretic (Sarić, 1989). In addition, *V. myrtillus* leaf infusions are traditionally used as a folk medicine treatment of diabetes, although recent studies show weak results (Helmstädter and Schuster, 2010). Urinary tract infections are among the most common bacterial infections acquired in the community and in hospitals. Treatment of these infections with antibiotics leads to a more rapid resolution of symptoms and is more likely to clear bacteriuria, but also selects for resistant uropathogens and commensal bacteria. So, it is advisable to seek alternative methods of prevention and treatment of urinary tract infections (Foxman, 2010).

Although there are papers on phytochemical analysis of leaves and fruits of this plant (Jaakola et al., 2002; Jaakola et al., 2004), the aim of this study was to determine and compare the antibacterial and antioxidant activity of different extracts of fruits and leaves of this plant collected on Borja Mountain (RS, Bosnia and Herzegovina, W. Balkans). The second aim of this paper was to determine the total phenol and flavonoid content in water, ethanol and ethyl acetate extracts using spectrophotometric methods.

MATERIALS AND METHODS

Plant material

In summer of 2009, ripe fruits and leaves of *V. myrtillus* were collected from natural populations on Borja mountain in the region of Teslić city in southeast Republic of Srpska, Bosnia and Herzegovina (position: 44°35′N, 17°35′E, altitude: 180.00 m, habitat: coniferous forest). Plants were identified and confirmed and voucher specimens were deposited at the Herbarium of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac. The collected plant material was air dried in darkness at ambient temperature (20°C). The dried plant material was cut up and stored in paper bags until needed.

Chemicals

Organic solvents and sodium hydrogen carbonate were purchased from Zorka pharma" Šabac, Serbia. Gallic acid, rutin hydrate and aluminium chloride hexahydrate (AlCl₃) were purchased from Acros Organics, New Jersey, USA. Chlorogenic acid, Folin-Ciocalteu phenol reagent and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Chemicals Co, St Louis, MO, USA.

Preparation of plant extracts

Prepared plant material (10g) was transferred to dark-coloured flasks with 200 ml of solvent (water, ethanol, ethyl acetate) and stored at room temperature. After 24 h, infusions were filtered through Whatman No. 1 filter paper and residue was re-extracted with equal volume of solvents. After 48 h, the process was repeated. Combined supernatants were evaporated to dryness under vacuum at 40°C using Rotary evaporator. The obtained extracts were kept in sterile sample tubes and stored at -20°C.

Determination of total phenolic contents in the plant extracts

The bilberry extracts were analyzed for total phenolics spectrophotometrically by the Folin-Ciocalteu procedure (Wootton-Beard et al., 2011). The reaction mixture was prepared by mixing 0.2 ml of methanolic solution of extract (1 mg/ml) and 1.5 ml of 1:10 Folin-Ciocalteu reagent dissolved in water. The mixture was allowed to equilibrate for 5 min and then mixed with 1.5 ml 6% $NaCO_3$ solution. After incubation for 90 min at room temperature in darkness, the absorbance of the mixture was read at 725 nm against a blank using spectrophotometer. The blank was prepared with methanol instead of extract solution. The samples were prepared in triplicate and the mean value of absorbance was obtained. The same procedure was repeated for gallic acid which was used for calibration of standard curve. Total phenol content is reported as gallic acid equivalents by reference to linear equation of the standard curve ($y = 0.008x + 0.0077$, $R^2 = 0.998$). Then, the total phenolic content was expressed as gallic acid equivalents in miligrams per gram of extract (mg GAE/g of extract).

Determination of flavonoid concentrations in the plant extracts

The concentrations of flavonoids was determined using spectrophotometric method with aluminium chloride (Quettier-Deleu et al., 2000). The sample contained 1 ml of methanolic solution of the extract in the concentration of 1 mg/ml and 1 ml of 2% $AlCl_3$ solution dissolved in methanol. The mixture was vigorously shaken, and after 10 min of incubation at room temperature, the absorbance *versus* a prepared blank was read at 430 nm using spectrophotometer. The samples were prepared in triplicate and the mean value of absorbance was obtained. Rutin was used as a standard for calibration of standard curve. The concentrations of flavonoids were calculated from the linear equation of standard curve ($y = 0.021x + 0.040$, $R^2 = 0.999$). Then, the concentrations of flavonoids were expressed as miligram of rutin equivalent per gram of extract (mg of RUE/g of extract).

Evaluation of DPPH scavenging activity

The ability of the plant extract to scavenge DPPH free radicals was assessed using the method described by Takao et al. (1994). The stock solution of the plant extract was prepared in methanol to achieve the concentration of 2000 µg/ml. Further, two-fold dilutions were made to obtain concentrations of 1000, 500, 250, 125, 62.5 µg/ml. Diluted solutions of extract (2 ml each) were mixed with 2 ml of DPPH methanolic solution (80 µg/ml). After 30 min in darkness at room temperature, the absorbance was recorded in a spectrophotometer at 517 nm. The control samples contained 2 ml of methanol added to 2 ml of DPPH solution. Chlorogenic acid was used as a positive control. The experiment was performed in triplicate. Scavenging activity is expressed as the inhibition percentage calculated using the following equation:

Scavenging activity (%) = 100 x [($A_{control}$ − A_{sample}) / $A_{control}$].

Table 1. Total phenolic contents and concentrations of flavonoids in fruits of *V. myrtillus* extracts.

Type of extract	Total phenolic content[1] (mg GAE/g of extract)	Flavonoid concentration[1] (mg RUE/g of extract)
Water	31.44 ± 0.17	5.20 ± 0.08
Ethanol	40.32 ± 0.24	10.06 ± 0.11
Ethyl acetate	99.34 ± 1.12	23.26 ± 0.21

[1]Values represent mean ± standard deviation.

Table 2. Total phenolic contents and concentrations of flavonoids from leaves of *V. myrtillus* extracts.

Type of extract	Total phenolic content[1] (mg GAE/g of extract)	Flavonoid concentration[1] (mg RUE/g extract)
Water	119.17 ± 0.52	43.08 ± 0.68
Ethanol	107.79 ± 1.23	81.98 ± 0.00
Ethyl acetate	66.76 ± 0.78	94.49 ± 3.61

[1]Values represent mean ± standard deviation

Where, $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the extract.

Test bacterial strains

Antibacterial activity of water, ethanol and ethyl acetate extract from dried fruits and leaves of *V. myrtillus* was tested against 30 strains of bacteria including ten strains of *Escherichia coli* (Mf-Ec1, Mf-Ec2, Mf-Ec3, Mf-Ec4, Mf-Ec5, Mf-Ec6, Mf-Ec7, Mf-Ec8, Mf-Ec9, Mf-Ec10), ten strains of *Enterococcus faecalis* (Mf-Ef1, Mf-Ef2, Mf-Ef3, Mf-Ef4, Mf-Ef5, Mf-Ef6, Mf-Ef7, Mf-Ef8, Mf-Ef9, Mf-Ef10) and ten strains of *Proteus vulgaris* (Mf-Pv1, Mf-Pv2, Mf-Pv3, Mf-Pv4, Mf-Pv5, Mf-Pv6, Mf-Pv7, Mf-Pv8, Mf-Pv9, Mf-Pv10). The *E. coli* strains and *P. vulgaris* strains represented Gram-negative bacteria. Bacterial strains of *E. faecalis* were Gram-positive. All clinical isolates were a generous gift from the Institute of Public Health, Banja Luka.

Suspension preparation

The original density of the bacterial suspension was 0.5 Mc Farland after which the additional dilution in saline at the proportion of 1:10 was made. The final concentration of the bacteria in the test tubes was 10^6 colony forming units (CFU)/ml.

Macrodilution method

The minimum inhibitory concentration (MIC) of the extracts was determined by the tube dilution method through the series of dilutions (NCCLS, 1997). In the test tubes filled with the Mueller Hinton broth, the solution of the extracts was added and the series of double dilutes was made. In each of the test tubes, the 100 µl of the suspension of the tested bacteria was added. The 24 h incubation at the temperature of 37°C was conducted. The minimum bactericidal concentration (MBC) is the lowest concen-

tration of the tested substance which has the bactericidal effect. These values were collected by inoculation of the Mueller Hinton agar with the test tube content; it was the content from the test tubes in which the MIC was found and all the test tubes had more than the MIC found. Amoxicillin was used as a positive control. Whereas the extracts were dissolved in 10% dimethyl sulfoxide (DMSO), solvent control test was performed to study the effects of 10% DMSO on the growth of bacterial strains. It was observed that 10% DMSO did not inhibit the growth of bacteria.

Statistical analysis

SPSS program was applied only when the mean was calculated. Data are presented as means ± standard deviations.

RESULTS

Total phenolic content and flavonoid concentrations

The results of total phenolic content in the plant extracts are presented in Tables 1 and 2. The total phenolic content was expressed as gallic acid equivalents and ranged from 31.44 to 99.34 mg GAE/g in the extracts of fruits. The extracts obtained from leaves of *V. myrtillus* were richer in phenolic active compounds than the extracts of the fruit. The water leaves extract had the highest phenolic content with 119.17 mg of GAE/g of extract. The summary of quantities of flavonoids identified in the tested extracts is shown in Tables 1 and 2. The concentration of flavonoids in various extracts of *V. myrtillus* was determined using spectrophotometric method with aluminium chloride. The content of flavonoids was expressed as rutin equivalent. Total flavonoid content
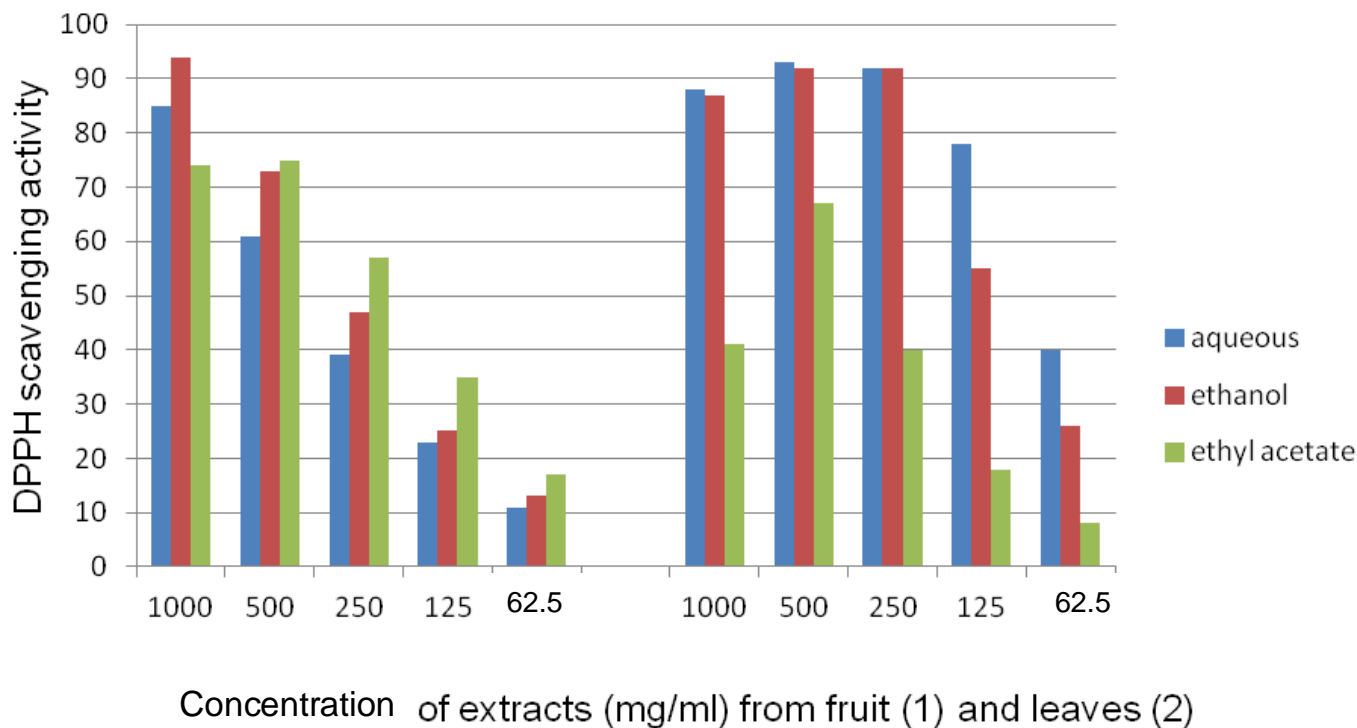
Figure 1. Antioxidant (DPPH scavenging, %RSA) activity of investigated fruit (1) and leaves (2) extracts of *V. myrtillus*

in plant extracts ranged between 5.20 to 94.49 mg RUE/g of extract. High concentrations of flavonoids were measured in ethyl acetate and ethanol extracts from leaves of *V. myrtillus*. Ethyl acetate is a low toxic solvent (Li at al., 2010).

Antioxidant activity

The antioxidant activity of six different extracts from *V. myrtillus* is expressed in Figure 1. The largest capacity in neutralization of DPPH radicals was measured in the ethanol extract from fruits of *V. myrtillus* and water extract from leaves of bilberry. In measuring total phenolic content, water extract showed the highest values. The extracts performing the highest antioxidant activity had the highest concentration of phenols.

Antibacterial activity

The results of *in vitro* antibacterial activities of water, ethanol and ethyl acetate extracts from fruits and leaves of *V. myrtillus* against 30 strains of Gram-positive and Gram-negative pathogenic bacteria are presented in Tables 3 and 4. Extracts from *V. myrtillus* inhibited several urinary pathogens extracted from urine samples. In general, the activity of extracts depended both on the species of bacteria and on the type and concentration of

extract and varied between 10 and 40 mg/ml. The ethanol and the ethyl acetate extract of fruits showed better activity than water extract in relation to strains of *E. faecalis* and *P. vulgaris*. The all tested extract from leaves showed similar activity against strains of *E. coli, E. faecalis* and *P. vulgaris*. The most sensitive strain of tested bacteria towards ethanol extracts of fruits and leaves of *V. myrtillus* was strain of *E. faecalis* MFBL-Ef8. On the other hand, strains of *E. coli* were the most resistant. All tested extracts demonstrated approximately similar activity in relation to the tested different strains of same bacteria.

DISCUSSION

Based on the obtained values of the concentration of flavonoids in the examined extracts of *V. myrtillus*, it was found that the highest concentration of these compounds was in the extracts obtained using solvents of moderate polarity. The concentration of flavonoids in plant extracts depends on the polarity of solvents used in the extract preparation (Min and Chun-Zhao, 2005), different locations from which the plant came and plant growing conditions.

The ethanol extract from fruits and water extract from leaves of *V. myrtillus* had high concentration of total phenols, which is in correlation with the intense antioxidant activity of extracts.

Table 3. Antibacterial activities of water, ethanol and ethyl acetate extracts from fruits of *V. myrtillus* against tested strains of bacteria based on macrodilution method.

Species	Aqueous extract		Ethanol extract		Ethyl acetate extract		Amoxicillin	
	MIC[1]	MBC[2]	MIC	MBC	MIC	MBC	MIC	MBC
E. coli Mf-Ec1	20	40	40	>40	40	>40	1000	2000
E. coli Mf-Ec2	20	40	40	>40	40	>40	4000	8000
E. coli Mf-Ec3	20	40	40	>40	40	>40	4	8
E. coli Mf-Ec4	20	40	40	>40	40	>40	2	4
E. coli Mf-Ec5	20	40	40	>40	40	>40	2000	4000
E. coli Mf-Ec6	20	40	40	>40	40	>40	2000	4000
E. coli Mf-Ec7	20	40	40	>40	40	>40	4	4
E. coli Mf-Ec8	20	40	40	>40	40	>40	1000	2000
E. coli Mf-Ec9	20	40	40	>40	40	>40	4000	8000
E. coli Mf-Ec10	20	40	40	>40	40	>40	1000	2000
E. faecalis Mf-Ef1	20	>40	10	20	10	20	0.977	125
E. faecalis Mf-Ef2	20	>40	10	20	10	20	0.488	>125
E. faecalis Mf-Ef3	20	>40	10	20	10	20	0.488	>125
E. faecalis Mf-Ef4	20	>40	10	20	10	20	0.488	>125
E. faecalis Mf-Ef5	20	>40	10	20	10	20	0.488	>125
E. faecalis Mf-Ef6	20	>40	10	20	10	20	0.488	>125
E. faecalis Mf-Ef7	20	>40	10	20	10	20	0.977	>125
E. faecalis Mf-Ef8	20	>40	5	10	10	20	0.488	125
E. faecalis Mf-Ef9	20	>40	10	20	10	20	0.488	>125
E. faecalis Mf-Ef10	20	>40	10	20	10	20	0.488	>125
P. vulgaris Mf-Ef1	20	40	10	20	10	40	>4000	>4000
P. vulgaris Mf-Ef2	20	40	10	20	10	40	>4000	>4000
P. vulgaris Mf-Ef3	20	40	10	20	10	40	>4000	>4000
P. vulgaris Mf-Ef4	20	40	10	20	10	40	0.977	7.812
P. vulgaris Mf-Ef5	20	40	10	20	10	40	>4000	>4000
P. vulgaris Mf-Ef6	20	40	10	20	10	40	0.977	7.812
P. vulgaris Mf-Ef7	20	40	10	20	10	40	>4000	>4000
P. vulgaris Mf-Ef8	20	40	10	20	10	40	2000	>4000
P. vulgaris Mf-Ef9	20	40	10	20	10	40	>4000	>4000
P. vulgaris Mf-Ef10	20	40	10	20	10	40	>4000	>4000

[1]Minimum inhibitory concentration (MIC) and [2]minimum bactericidal concentration (MBC) values are given as mg/ml for plant extracts and µg/ml for antibiotic.

Berry fruits are very rich sources of bioactive compounds as phenolics and organic acid. Comparison of the antibacterial effects of extracts from fruits and extracts from leaves of *V. myrtillus* showed that phenolic compounds were only partially responsible for the growth inhibition of bacterial strains and most of the antibacterial effects probably originated from other compounds such as organic acids.

Previous studies support our research data to a great extent (Puupponen-Pimia et al., 2005b; Badjakov et al., 2008). Differences in the results related to some strains of bacteria can also be explained by different sensitivity of tested species of bacteria, different methods of testing and the solvents used. In previous study, Puupponen-Pimia et al. (2005a) investigated the antimicrobial activity

of extracts from fruits of bilberry on *Salmonella enterica* and *Staphylococcus aureus* and determined that *V. myrtillus* possess clear antimicrobial effects on these bacteria.

E. coli is a bacterium that is commonly found in the intestine. Most *E. coli* strains are commensals. However, some strains can cause severe disease (Brzuszkiewicz et al., 2011). Enterococci are Gram-positive commensals of the gastrointestinal tract of humans. *E. faecalis* is an important cause of infections in hospitalized, immunocom-promized patients (Schaik et al., 2010). *Proteus* species have an important place in environmental pollution bioremediation. *Proteus* sp. from various environments are able to utilize and degrade many variety of toxic materials. Many researches have reported the potential

Table 4. Antibacterial activities of water, ethanol and ethyl acetate extracts from leaves of *V. myrtillus* against tested strains of bacteria based on macrodilution method.

Species	Aqueous extract		Ethanol extract		Ethyl acetate extract		Amoxicillin	
	MIC[1]	MBC[2]	MIC	MBC	MIC	MBC	MIC	MBC
E. coli Mf-Ec1	40	>40	40	>40	20	40	1000	2000
E. coli Mf-Ec2	40	>40	40	>40	20	40	4000	8000
E. coli Mf-Ec3	40	>40	40	>40	20	40	4	8
E. coli Mf-Ec4	40	>40	40	>40	20	40	2	4
E. coli Mf-Ec5	40	>40	40	>40	20	40	2000	4000
E. coli Mf-Ec6	40	>40	40	>40	20	40	2000	4000
E. coli Mf-Ec7	40	>40	40	>40	20	40	4	4
E. coli Mf-Ec8	40	>40	40	>40	20	40	1000	2000
E. coli Mf-Ec9	40	>40	40	>40	20	40	4000	8000
E. coli Mf-Ec10	40	>40	40	>40	20	40	1000	2000
E. faecalis Mf-Ef1	40	>40	20	40	40	>40	0.977	125
E. faecalis Mf-Ef2	40	>40	20	40	40	>40	0.488	>125
E. faecalis Mf-Ef3	40	>40	20	40	40	>40	0.488	>125
E. faecalis Mf-Ef4	20	>40	20	40	40	>40	0.488	>125
E. faecalis Mf-Ef5	40	>40	20	40	40	>40	0.488	>125
E. faecalis Mf-Ef6	40	>40	20	40	40	>40	0.488	>125
E. faecalis Mf-Ef7	20	>40	20	40	40	>40	0.977	>125
E. faecalis Mf-Ef8	20	>40	20	20	40	>40	0.488	125
E. faecalis Mf-Ef9	40	>40	20	40	40	>40	0.488	>125
E. faecalis Mf-Ef10	20	>40	20	40	40	>40	0.488	>125
P. vulgaris Mf-Ef1	20	>40	20	40	40	>40	>4000	>4000
P. vulgaris Mf-Ef2	20	>40	20	40	40	>40	>4000	>4000
P. vulgaris Mf-Ef3	20	>40	20	40	40	>40	>4000	>4000
P. vulgaris Mf-Ef4	20	>40	20	40	40	>40	0.977	7.812
P. vulgaris Mf-Ef5	20	>40	20	40	40	>40	>4000	>4000
P. vulgaris Mf-Ef6	20	>40	20	40	40	>40	0.977	7.812
P. vulgaris Mf-Ef7	20	>40	20	40	40	>40	>4000	>4000
P. vulgaris Mf-Ef8	20	>40	20	40	40	>40	2000	>4000
P. vulgaris Mf-Ef9	20	>40	20	40	40	>40	>4000	>4000
P. vulgaris Mf-Ef10	20	>40	20	40	40	>40	>4000	>4000

[1]Minimum inhibitory concentration (MIC) and [2]minimum bactericidal concentration (MBC) values are given as mg/ml for plant extracts and μg/ml for antibiotic.

biodegradation of xenobiotics by the members of *Proteus* genus (Ceyhan, 2012).

The results of our research indicate good antibacterial activity of fruits of *V. myrtillus*. Thus, fruits and leaves of *V. myrtillus* should be considered a potential source of antibacterial substances. Anthocyanins and phenolic acid derivates were identified in previous investigations on *V. myrtillus*. Several studies demonstrated strong antioxidant activity of these phenolic compounds (Nakajima et al., 2004; Viljanen et al., 2004; Ehala et al., 2005). Recent studies confirm these findings also for phenolic composition and antioxidant capacity of bilberry leaves (Martz et al., 2010).

Literature data indicate that the *V. myrtillus* is a medicinal plant in traditional medicine and it is applied as a source of active substances (Taruscio et al., 2004;

Faria et al., 2005; Cooke et al., 2006). Leaves of *V. myrtillus* can prevent urinary tract infections. Fruits of bilberry have been used for the treatment of urinary tract infections.

Conclusions

The results of this research suggest that water, ethanol and ethyl acetate extracts of *V. myrtillus* inhibit the growth of human pathogens and can have significant effect on the prevention of the urinary tract infection. Antibacterial compounds from *V. myrtillus* may have important applications as natural antibacterial agents. Therefore, the fruits and leaves of this plant are natural sources of antioxidant substances of high importance.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Development of anaerobic consortia and its invitro evaluation for biomethanation potential of coffee processing wastes

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An anaerobic consortia was prepared by using *Clostridium* strains already screened for biocatalytic ability to degrade cellulose, pectin and tannins together with *Methanosarcina* sp. Among the two inoculum sources studied, the reactor having anaerobic consortium could ferment coffee pulp waste (CPW) and coffee processing waste water (CPWW) leading to 67% soild removal, 62.27% pectin, 27.4% tannins, 63.52% cellulose and 60% hemicellulose reduction. This resulted in total biogas production of 0.017 m^3 over a period of eight weeks and highest methane content of 65% at 6th week when compared to cow-dung slurry. The bioenergy obtained was converted into electricity units saving 27.2% of power consumption with anaerobic consortium for a small scale coffee processing unit generating 5 ton of coffee pulp waste. This study indicates that anaerobic consortium with efficient microbial strains appeared to be a promising technology for mitigating the present problems caused by coffee processing wastes.

Key words: Anaerobic consortium, biogas, methane, total solids (TS) removal, biopolymers.

INTRODUCTION

Anaerobic biological system offers the greatest potential for the treatment of industrial effluent. Anaerobic decomposition of complex organic matter into methane and carbon dioxide is a complex process, involving a well-organised community of several microbial populations. The flow pattern and the formation of intermediate metabolites during degradation depend on the microbial status and the operating conditions. Biogas production through anaerobic digestion of biomass including the organic fraction of waste materials and residues is a particularly promising choice and experiences increasing interest worldwide (Onodera et al., 2012). The anaerobic degradation of organic polymers is a multistage process such as hydrolysis, acidogenesis, acetogenesis and methanogenesis. These processes do not have high energy demand resulting in low biomass production and generation of methane, which can be used as an energy source (Ma and Ong, 1988).

Coffee processing industries emanate huge volumes of wastes in the form of coffee pulp and coffee processing waste water. Coffee pulp waste containing sizable proportion of compounds like pectin (6.5%), reducing sugars (12.4%), non reducing sugars (2.0%), caffeine (2.3%), chlorogenic acid (2.6%), lignin, cellulose etc. could be digested anaerobically to produce biogas. Coffee processing waste water (CPWW) is loaded with high organic matter rather than its inherent toxicity. The major constituents of CPWW are suspended solids and

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dissolved solids containing pectin, proteins and sugars which are biodegradable in nature. The concentrations of these pollutants vary with the quantity of water used for processing of fruits (Shanmukhappa et al., 1998). During the fermentation process in wastewater, the acidification of sugars will drop the pH to around 4 or less, and the digested mucilage will be precipitated out of solution and will build a thick crust on the surface of the wastewater. At around pH 7 and over, flavanoids turn wastewater into dark green to black colour staining rivers downstream from coffee factories. Considering the serious impact of coffee processing wastes to the environment, anaerobic digestion holds an alternate low cost energy conserving mitigation.

The total solids and the volatile solids have been reduced considerably during the anaerobic digestion of coffee pulp. Methane production from coffee wastes has been enhanced by the addition of cowdung and old slurry of a biogas plant. The potential daily yield of biogas from 2 ton of coffee pulp is approximately 131 m^3 equivalent in terms of its fuel value to 100 lts of petrol (Houbron et al., 2003). Maximization of biogas production has also been undertaken (Boopathy and Mariappan, 1984) when coffee waste, cowdung and old slurry were mixed in the ratio of 3:1:1. Efficient treatment of coffee processing waste water employing cowdung slurry as inoculum have been reported by Selvamurugan et al. (2010).

Cellulolytic bacteria and Methanogenic Archaae represents one of the components of cowdung slurry or old digested slurry or rumen fluid. Screening of efficient strains from these sources can be effectively utilized for sustainable degradation of lignocellulosic biomass degradation and thereby biofuel generation. Till date only cowdung slurry, digester slurry or goat rumen is widely employed as inoculum source for anerobic digestion of coffee wastes.

A definite paucity of information exists regarding the pectinolytic behavior of cellulolytic *Clostridium* sp and syntrophic association of *Clostridium* sp with Methanogenic archae. Our efforts would be directed towards developing anerobic consortia using cowdung slurry as matrices and co-inoculating with well characterized *Clostridium* sp involved in biodegradation of polymers and methanogenic archae as partners in the association.

MATERIALS AND METHODS

Growth and maintenance of the strains

A set of five anaerobic isolates and five methanogenic archae were isolated from coffee processing wastes by enrichment technique using goat rumen fluid. The enrichment technique was used to enhance the population of anaerobic bacteria involved in bioconversion, since the native population might be low in the coffee wastes. The coffee pulp and coffee processing waste water were mixed with goat rumen fluid in equal proportion under anaerobic condition and incubated at 37°C for two weeks. Anaerobic bacteria were isolated by Hungate's roll tube technique using specific selective media. The anaerobic isolates were screened

for their biodegradation potential of complex biopolymers vis., cellulose, pectin, tannic acid, and hemicelluloses represented by coffee processing wastes. The elite strains TCW 3 and TCW 5 for efficient biodegradation belonging to the genus *Clostridium* were maintained in modified Hungates's medium (Ramasamy et al., 1992). The exponential phase cultures (12-15 days), incubated at 35+/- 2°C were used for further studies.

The methanogenic archae was maintained in Mah medium with acetate (Mah et al., 1978) and were screened based on their ability for methanogenesis and methane recovery. The selected strain *Methanosarcina* sp. (TCWMS 5) was used after repeated subculturing in Mah medium and was grown under anaerobic condition at 35+/- 2°C.

Co-cultivation of Clostridium and Methanosarcina sp.

To a two welled anaerobic glass container, 50 ml of sterile CPWW and 25 g of sterile CPW was transferred to the main well and 10 ml of sterile CPWW and 5 g of sterile CPW was transferred to the test tube well. The two mouths of the glass container were sealed with a butyl rubber stopper and aluminium cap.

The contents were sterilized at 121°C for 10 min and allowed to cool. The main well was inoculated with 5 ml of fresh cells (10^6/ ml) of *Clostridium* sp as per the experimental details mentioned below through disposable needles and syringes in N_2 atmosphere. In the test tube well, 2 ml of *Methanosarcina* sp. (TCWMS 5) was inoculated and incubated at 37°C.

The methane content of the gas phase in the anaerobic glass container was measured after the third day by KOH displacement method using saccharometer. Residual cellulose, pectin and tannic acid were also assessed using standard procedures.

Arrangement of experiment for co-culture studies

The arrangement of experiment for co-culture studies included: T_1, *Clostridium* sp. TCW 3 + *Methanosarcina* sp. TCWMS 5 + SCPW +SCPWW; T_2, *Clostridum* sp. TCW 3 + *Methanosarcina* sp. TCWMS 5 + SCPW +SCPWW; T_3, *Clostridium* sp. TCW 3 alone in the main well + SCPW +SCPWW; T_4, *Clostridium* sp. TCW 3 alone in the main well + SCPW +SCPWW; T_5, *Methanosarcina* sp. TCWMS 5 alone in the test tube well + SCPW +SCPWW; T_6, both wells uninoculated + SCPW +SCPWW

Development of anaerobic consortia

The strains *Clostridium* sp. (TCW 3 and TCW 5) and *Methanosarcina* sp. (TCWMS 5) were mass multiplied in their respective media. Optimization of inoculum rate of the three partners was undertaken by using 2-10% anaerobic cultures vis a vis 2-10% of methanogenic archae in sterile cowdung slurry as a matrice for multiplication.

After one week of growth under optimum conditions, samples were drawn periodically at weekly interval and examined for their population. Microscopic analysis was carried out to study the survival and colonization All the cultures inoculated at the rate of 10% was found to be optimum showing maximum number of colonies.

Invitro evaluation of anaerobic consortia in bench scale fermentation of coffee wastes

A bench scale anaerobic fermentation experiment was set up to assess the performance of anaerobic consortia interms of biocatalysis and methanogenesis using coffee wastes viz., CPW and CPWWW as substrates. The coffee pulp was made into slurry
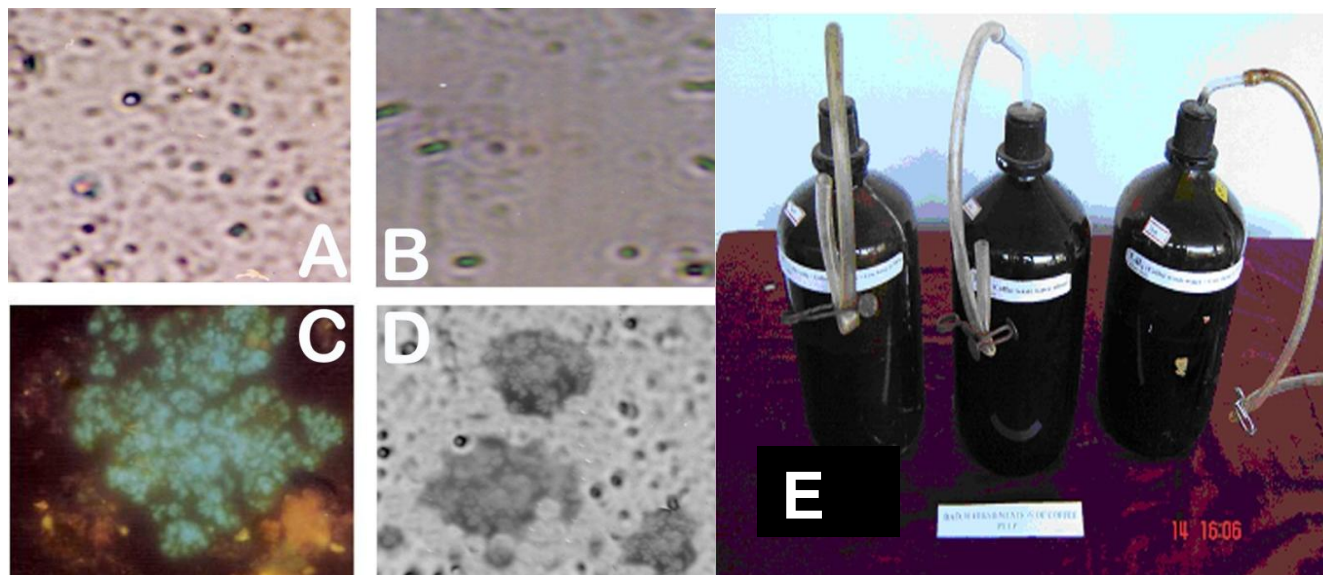
Figure 1. A, *Clostridium* sp. TCW 3; **B,** *Clostridium* sp. TCW 5; **C,** *Methanosarcina* sp. **D,** Microscopic view of anaerobic consortium. E, Experimental set up for bench scale anaerobic fermentation of coffee processing wastes

with coffee processing waste water (250 g of CPW and 1000 ml of CPWW)

so as to have a TS per cent of 25%. Amber colored glass containers of 2.5 L capacity were filled with coffee pulp slurry as per the experimental details given below. The performance of anaerobic consortia was compared with cowdung slurry from an active cow dung based plant. Both the inoculums were used at a rate of 10 per cent of the substrate volume. Only 2/3 of the bottles were filled with slurry and inoculum and 1/3 of the space was allowed for biogas collection (Figure 1E).

An assembly of bent glass tube fixed tightly on rubber cork and attached to rubber tubes with a pinch cork was used to create airtight condition. Each treatment was replicated thrice and the biogas generated was recorded daily by water displacement method and the methane content was quantified in a Gas Chromatograph with thermal conductivity detector (TCD) having 'Porapak Q' column by setting the oven temperature at 80 to 100°C, injector temperature at 100 to 200°C, detector temperature at 120°C and using nitrogen as carrier gas at a flow rate of 30 ml min^{-1}.The change in total solids, pH, EC and the degradation of biopolymers such as pectin, cellulose, hemi cellulose and protein were assessed at periodic intervals (APHA, 1992).

Experimental set-up for bench scale anaerobic fermentation

Experimental set-up for bench scale anaerobic fermentation included: T_1, Coffee pulp alone (CPW); T_2, Coffee waste water alone (CWW);T_3, CPW + CWW; T_4, CPW + cow dung slurry (10%); T_5, CWW + cow dung slurry (10%); T_6, CPW + CWW + cow dung slurry (10%); T_7, CPW + Anaerobic consortia (10%); T_8, CWW + Anaerobic consortia (10%); T_9, CPW + CWW + Anaerobic consortia (10%)

Statistical analysis

The data recorded in triplicates for the growth and biochemical parameters in selected treatments were subjected to ANOVA (Analysis of Variance) in accordance with the experimental design (completely randomize block design) using SPSS.10 statistical package were used to quantify and evaluate the source of variation.

RESULTS

A set of five anaerobic strains isolated from coffee processing wastes by enrichment technique were screened in terms of biocatalysis and growth on different substrates viz., cellulose, pectin and tannin. Two strains identified as *Clostridium* sp (TCW 3 and TCW 5) exhibited more efficiency in biodegrading cellulose and pectin, tannic acid respectively on 12th day. The combination of both strains performed significantly in terms of percent reduction of cellulose, pectin and tannic acid rather than individual strains (data not shown). A gradual increase in VFA like acetate upto 12 days, and propionate upto six days was observed, however VFA declined thereafter. The five archael strains were screened based on cumulative biogas production and methane generation. The strain TCWMS 5 reported significant biogas production ability and methane recovery. The microscopic analysis confirmed TCWMS 5 as well distinct sarcina type of cells (Figure 1C).

Co-culturing of *Clostridium* sp. and *Methanosarcina* sp.

Considerable work has been available on the cellulolytic behavior of *Clostridium* sp but its quiet rare to obtain a *Clostridium* strain with the ability to degrade pectin and tannins in addition to cellulose. Co-culturing of *Clostridium* sp. and *Methanosarcina* sp. resulted in significant reduction of pectin content. On the 8th week, maximum

Table 1. Per cent reduction of biopolymers on 8th week of coculturing.

Treatment	Pectin (%)	Tannin (%)	Cellulose (%)
T_1	42.62	28.95	56.35
T_2	54.1	47.37	44.44
T_3	31.15	23.68	47.09
T_4	36.07	36.81	37.83
T_5	14.75	5.26	6.08
T_6	8.19	7.89	3.44
SEd	0.095	0.099	0.123
CD(0.05)	0.208	0.217	0.267

T_1, TCW 3 + TCW MS 5 + SCPW + SCPWW; T2, TCW 5 + TCW MS 5 + SCPW + SCPWW; T_3, TCW 3 alone in the main well + SCPW +SCPWW ;T_4, TCW 5 alone in the main well + SCPW +SCPWW ; T_5, TCW MS 5 alone in the test tube well + SCPW + SCPWW; T_6, Both wells uninoculated + SCPW +SCPWW. SCPW, sterilized coffee pulp waste; SCPWW, sterilized coffee processing waste water. *Values are average of three replications.

Table 2. Cumulative biogas production during co-culturing.

Treatment/ week	Cumulative biogas production (ml/week)								
	I	II	III	IV	V	VI	VII	VIII	Total
T_1	45	80	120	185	220	245	230	185	1310
T_2	55	85	105	158	196	242	215	165	1221
T_3	-	-	20	35	55	40	-	-	150
T_4	-	-	25	40	65	50	-	-	180
T_5	-	-	55	88	105	125	85	65	523
T_6	-	-	-	15	25	40	35	20	135

T_1, TCW 3 + TCW MS 5 + SCPW + SCPWW; T2, TCW 5 + TCW MS 5 + SCPW + SCPWW; T_3, TCW 3 alone in the main well + SCPW +SCPWW; T4, TCW 5 alone in the main well + SCPW +SCPWW ; T_5, TCW MS 5 alone in the test tube well + SCPW + SCPWW; T_6, Both wells uninoculated + SCPW +SCPWW. SCPW, Sterilized coffee pulp waste; SCPWW, sterilized coffee processing waste water. *Values are average of three replications.

reduction in pectin content (54.1%) was observed with *Clostridium* sp. TCW 5 and *Methanosarcina* sp. (TCWMS 5) followed by *Clostridium* sp. TCW 3 and *Methanosarcina* sp. (TCWMS 5). The tannic acid content reduced towards the period of incubation and the reduction per cent varied significantly among all the treatments. Co- culturing of *Clostridium* sp. TCW 5 and *Methanosarcina* sp. (TCWMS 5) recorded maximum reduction of tannic acid (47.37 per cent) followed by *Clostridium* sp. TCW 5 alone. For cellulose degradation, *Clostridium* sp. TCW 3 with *Methanosarcina* sp. (TCWMS 5) recorded maximum reduction of cellulose (56.35%) followed by *Clostridium* sp. TCW 3 alone (47.09%). The results are presented in Table 1.

Co-culturing and Methanogenesis

During co-culturing, an increasing trend of biogas production was noticed upto the 6th weeks in all the treatments (Table 2). Maximum biogas production was recorded by *Clostridium* sp. TCW 3 and *Methanosarcina* sp. TCWMS 5 (1,310 ml) followed by *Clostridium* sp.

TCW5 and *Methanosarcina* sp. TCWMS 5 (1,221 ml). The results opened new platform of bringing the two strains of Clostridium sp with the ability to degrade complex polymers viz., cellulose, hemicelluloses, pectin tannins and *Methanosarcina* sp. which could increase the efficiency of anaerobic fermentation.

Development of anaerobic consortium and its survival studies

The population of the inoculated strains *Clostridium* sp and *Methanosarcina* sp. was enumerated in their respective media before inoculation and monitored to evaluate the viability of the cells. Despite a 10-25% reduction in the number of cells after one week, the cells remained static till 3 weeks and declined later (Figure 2). The bacterial cultures showed a similar level of reduction with time. Protein accumulation also showed a drastic reduction after 3 weeks (data not shown). The viability and survival of the strains in the consortium were also confirmed by microscopic analysis (Figure 1A, B, C and D).
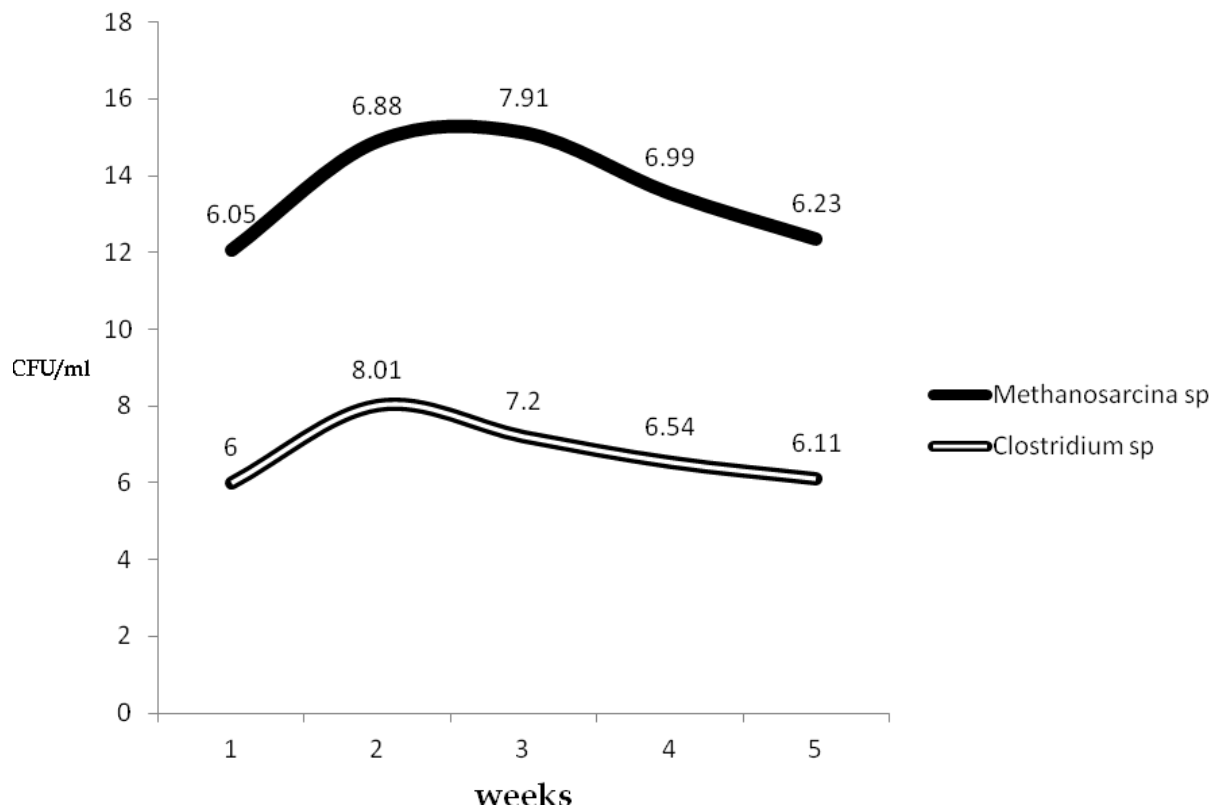
Figure 2. The log CFU/ml of inoculated strains at different intervals.

Invitro evaluation of anaerobic consortium for anaerobic fermentation of coffee processing wastes

Effect of anaerobic consortium on pH and EC

The anaerobic fermentation process is known to be extremely sensitive to pH. During the course of the study, pH gradually decreased on 30th day and increased on 60th day irrespective of all the treatments. The decrease in pH during fermentation may be due to the production of volatile fatty acids and later the pH increased because of methanogenic activity (Table 3). Data showed that EC declined gradually during anaerobic fermentation and maximum reduction was observed in treatments with 10% anaerobic consortium as inoculums rather than cowdung slurry 10%. This represents a good operation condition of the system which favours hydrolysis, acidogenesis followed by acetogenesis and methanogenesis.

Effect of anaerobic consortium on solid removal

The total solids (TS) have been reduced considerably during anaerobic fermentation of coffee pulp waste. In reactor with anaerobic consortium as inoculum source, on 60th day, the TS of the waste water and coffee pulp waste were 7.1 [1] and 90.5 g L^{-1} respectively. A maximum TS removal of 67% was achieved by CPW + CPWW + anaerobic consortium (10%). Solid removal efficiency was very poor in the reactors without any inoculum source. In reactor with cowdung slurry as inoculum the TS were 9.2 and 113.5 g L^{-1} respectively for coffee waste water and coffee pulp waste on 60th day. The TS removal efficiency with cowdung slurry was only 59.68%. At each stage, the total solids decreased and total biogas production increased due to faster metabolic rate of the organisms.

Effect of anaerobic consortium on biopolymers pectin, tannin, cellulose, hemicellulose

The biopolymers like pectin, tannic acid, cellulose, hemicellulose, proteins and reducing sugars were significantly reduced during anaerobic fermentation of coffee pulp waste. In the reactor with anaerobic consortium, pectin and tannic acid content were reduced by 62.27 and 27.94% respectively (Table 4). The per cent reduction of cellulose and hemicellulose were 63.52 and 60% respectively. But with cowdung slurry, only 51.25, 15.94, 47.44 and 45.85% reduction in pectin, tannin, cellulose and hemicellulose respectively was observed.

Table 3. Change in pH and EC during bench scale anaerobic fermentation of coffee processing wastes.

Treatment	pH			EC (dSm^{-1})		
	Initial	30th day	60th day	Initial	30th day	60th day
T_1 : Coffee pulp waste alone (CPW)	6.01	5.86	6.22	0.78	0.73	0.66
T_2 : Coffee waste water alone (CPWW)	4.50	4.41	4.63	0.98	0.95	0.91
T_3 : CPW + CPWW	6.33	6.06	6.52	1.5	1.2	1.0
T_4 : CPW + CD (10%)	6.54	6.07	7.03	1.3	1.1	0.95
T_5 : CPWW + CD (10%)	4.87	4.51	5.15	1.2	1.1	0.93
T_6 : CPW + CPWW + CD (10%)	6.66	6.44	7.11	1.8	1.3	0.90
T_7 : CPW + AC (10%)	6.52	6.10	7.18	1.7	1.3	0.85
T_8 : CPWW + AC (10%)	4.91	4.64	5.25	1.1	0.89	0.78
T_9 : CPW + CPWW + AC (10%)	6.73	6.34	7.23	2.1	1.1	0.70

Table 4. Per cent reduction of biopolymers during bench scale anaerobic fermentation of coffee processing wastes.

Treatment	% reduction in biopolymers on 60th day						
	Total solid	Pectin	Tannin	Cellulose	Hemicellulose	Protein	Reducing sugar
T_1 : Coffee pulp waste alone (CPW)	9.96	11.15	3.90	13.60	16.39	7.21	12.40
T_2 : Coffee waste water alone (CPWW)	8.44	17.20	5.68	14.44	8.04	8.57	6.10
T_3 : CPW + CPWW	13.8	14.08	5.86	20.77	16.81	11.51	12.60
T_4 : CPW + CD (10%)	54.56	44.29	11.11	42.00	38.79	42.95	36.40
T_5 : CPWW + CD (10%)	46.51	43.07	10.90	46.22	38.46	45.41	30.00
T_6 : CPW + CPWW + CD (10%)	59.68	51.25	15.94	47.44	45.85	53.55	42.20
T_7 : CPW + AC (10%)	63.80	58.64	21.21	59.87	57.73	55.63	50.00
T_8 : CPWW + AC (10%)	61.20	54.74	18.95	57.14	58.82	54.80	54.20
T_9 : CPW + CPWW + AC (10%)	67.00	62.27	27.94	63.52	60.00	62.88	57.20
SED	0.3080	0.0702	0.0860	0.0893	0.0755	0.0812	0.0159
CD (0.05%)	0.6471	0.1476	0.1806	0.1876	0.1586	0.1706	0.0333

Effect of anaerobic consortium on protein and reducing sugars

The protein content was found to decrease gradually during anaerobic digestion of coffee processing waste and maximum reduction of protein content (62.88%) was observed in reactor with CPW + CPWW and anaerobic consortium (10 %) followed by reactor with CPW and anaerobic consortium (55.63 %) on 60th day of fermentation. Reducing sugar content in all treatments increased during the first phase of fermentation and thereafter decreased. In the initial phase, the level of reducing sugar in the treatments ranged from 0.33 to 1.45%. At 30th day of anaerobic fermentation, all the treatments showed increased level of reducing sugars but at 60th day, significant reduction was observed in all the treatments. Maximum reduction of 57.2% was observed in the reactor with CPW + CPWW and anaerobic consortium 10% (Table 4).

Effect of anaerobic consortium on methanogenesis and methane recovery

As the fermentation proceeds, the volume of biogas and methane produced also increased. The performance of the reactor with anaerobic consortium was much better than cowdung slurry, on 6th week with maximum TS reduction of 67 % and the average biogas production was 5.5 m^3 (Figure 4). The methane content was 65% on 6th week and declined gradually. This increasing trend was noticed up to sixth week in reactors with 10% anaerobic consortium as inoculum and up to seventh week in reactors with 10% cow-dung slurry.

Efficiency of anaerobic fermentation of coffee processing wastes

The efficiency of anaerobic fermentation of coffee proces-

Table 5. Efficiency of bench scale anaerobic fermentation of coffee processing wastes.

Treatment	Total gas (l)	Biogas/gTS added (l/g)
T_1 : Coffee pulp waste alone (CPW)	2.16	0.087
T_2 : Coffee waste water alone (CPWW)	1.24	0.805
T_3 : CPW + CPWW	3.65	0.146
T_4 : CPW + CD (10%)	10.46	0.418
T_5 : CPWW + CD (10%)	10.68	6.21
T_6 : CPW + CPWW + CD (10%)	12.83	0.510
T_7 : CPW + AC (10%)	12.87	0.515
T_8 : CPWW + AC (10%)	14.03	7.66
T_9 : CPW + CPWW + AC (10%)	16.74	0.669

CPW, Coffee pulp waste; CPWW, coffee processing waste water; CD, cow dung slurry; AC, Anaerobic consortia.

sing wastes was studied in terms of total biogas produced per gram of TS added. In this regard, coffee processing waste water with anaerobic consortium 10% and cowdung slurry as inoculum produced 7.66 and 6.21 L of biogas per gram of TS added and with coffee pulp waste, 0.515 and 0.418 L per gram of TS added respectively. The reactor with both CPW and CPWW inoculated with anaerobic consortium and cowdung slurry recorded 0.669 and 0.418 L of biogas per gram of TS added respectively (Table 5).

Change in microbial dynamics during anaerobic fermentation of coffee processing wastes

The population of total anaerobes, cellulolyzers and acid formers were found to increase up to 30th day of anaerobic digestion and afterwards recorded a gradual decline. Among the treatments, T_9 with CPW, CPWW and anaerobic consortium 10%, registered maximum population of total anaerobes, cellulolyzers and acid formers of about 43×10^5, 30×10^3 and 24×10^2 CFU ml^{-1} respectively followed by T_7 with CPW with anaerobic consortium 10% (28×10^5, 23×10^3 and 19.0×10^2 CFU ml^{-1} respectively) on 30th day of anaerobic fermentation. On the other hand, the population of methanogens recorded a gradual increase and reached maximum at 60th day of anaerobic digestion. In general the maximum population of methanogens was recorded in treatments receiving anaerobic consortium as inoculum followed by cow dung slurry. Similar to other anaerobes, T_9 (CPW, CPWW and anaerobic consortium 10%) registered maximum population of total methanogens (28×10^4 CFU ml^{-1} of sample) followed by T_7 with CPW with anaerobic consortium10 per cent (25×10^4 CFU ml^{-1} of sample) on 60th day of anaerobic fermentation.

Techno-economic feasibility of anaerobic consortium with cowdung slurry for anaerobic fermentation of coffee processing wastes

The Techno-economic feasibility of anaerobic consortium with cowdung slurry for anaerobic fermentation of coffee processing wastes is shown in Table 6. So, 27.2% of the expenditure on electricity can be saved by using biogas as fuel generated from coffee pulp wastes employing anaerobic consortium.

DISCUSSION

Co-culturing and Methanogenesis

Clostridium sp. TCW 3 and TCW 5 associated with *Methanosarcina* sp. TCWMS 5 under co-culture condition resulted in significant reduction of pectin, tannic acid and cellulose and generated significant amount of biogas. The co-culture of *Clostridium* sp. with *Methanosarcina* sp. accumulates methane with partial utilization of H_2 involved.

The results show that *Clostridium* sp. (TCW 5) possess a pectin methyl esterase that catalyses the hydrolysis of methyl esters linked with the production of methanol and pectic acids. The production of methanol and isopropanol was proportional to the amount of pectin introduced. Similarly, *Clostridium* sp. is able to ferment sugars into isopropanol via acetone-butanol fermentation. Since *Methanosarcina* sp. is a methylotrophic methanogen, it can reduce methanol to methane using the small amount of H_2 produced during the hydrolysis of pectin and cellulose. This shows the existence of interspecies hydrogen transfer and is in accordance with the findings of Ollivier and Garcia (1990). They reported that more than 95% of the methane can be obtained only by associating *C. thermocellum* with *Methanosarcina* sp. In the presence of methylotrophic *Methanosarcina* sp., methanol was reduced to methane without effect on pectin hydrolysis and a small amount of the H_2 produced was also used to reduce methanol. Studies on mehanogenic fermentation have been reported in mesophilic conditions for a bacterial co-culture by Rhode et al. (1981).

The presence of interspecies hydrogen transfer in the system demonstrated a well marked syntrophic association between the two partners which is a good indi-

Table 6. Techno-economic feasibility of anaerobic consortium with cowdung slurry for anaerobic fermentation of coffee processing wastes.

Detail	Anaerobic consortium (10%)	Cow dung slurry (10%)
Total biogas produced per 250 grams of coffee processing wastes	16.74 L (0.017 m^3)	12.83 L (0.013 m^3)
Total biogas per Kg of coffee processing wastes	0.068 m^3	0.052 m^3
Total biogas per ton of coffee processing wastes	68 m^3	52 m^3
For coffee processing unit generating 5 tonnes of wastes with an approximate power consumption of 2000 KW hr units		
Biogas from 5 tonnes of coffee processing wastes	340 m^3	260 m^3
1 m^3 biogas = 1.6 KW hr units		
Biogas from 5 tonnes of coffee processing wastes equivalent to	544 KW h units	416 KW h units
Revenue at of Rs 30/KW h unit	Rs.16,320	Rs. 12,480
Cost of electricity (for 2000 KW h units at Rs.30/KW hr unit)	Rs.60,000	Rs. 60,000
Net saving interms of power (%)	27.2	20.8

cator of the promise of this anaerobic consortium. Though the pH of the system dropped down to acidic during the course of fermentation, the methanogenic activity is maintained due to the existence of interspecies hydrogen transfer within the community. There would have exhibited a shift in acetate degradation from direct acetate cleavage towards syntrophic acetate oxidation coupled with hydrogenotrophic methanogens as described earlier (Hao et al., 2013). The syntrophic acetate oxidizing (SAO) bacteria and hydrogenotrophic methanogens (HM) including *Methanomicrobiales* and *Methanobacteirales* were more tolerant to the stress from high acetate concentration and high pH. Resumption from alkali condition to normal pH stimulated the growth of acetate oxidizing syntrophs. These results point to the possibility to regenerate the deteriorated anaerobic digesters by addition of acclimatized inocula rich in acetate-oxidizing syntrophs.

Development of anaerobic consortium and its survival studies

For a successful consortium, the performance as well as the survival of the organisms in a single new platform is very important. Static population of the individual strains upto three weeks showed that significant level of compatability exists among the individual strain which is a mandate for developing any microbial consortium. The results reveal that positive interactions existed among the anaerobic bacteria and the archae. Although there was a dissimilar pattern of reduction in the populations of individual bacterial cultures, which can be attributed to the intrinsic growth characteristics, the comparatively lower counts illustrated the time taken for initial acclimatization (lag phase) and the loss of viability at much later stages.

Efficiency of anaerobic consortium for anaerobic fermentation of coffee processing wastes

During anaerobic fermentation, gradual decline in EC was noticed and maximum reduction was observed in treatments with 10% anaerobic consortium as inoculums. This represents a good operation condition of the system which favours hydrolysis, acidogenesis followed by acetogenesis and methanogenesis. TS reduction of 67% was recorded by the treatment imposed with the developed anerobic consortium rather than cowdung slurry which is conventionally used as an inoculums source. This shows faster metabolic rate of the individual strains in the anaerobic consortium than generalized microbes in the cowdung slurry.

The reduction of biopolymers during anaerobic fermentation of coffee pulp waste might be due to the conversion to simple monomers like glucose and maltose in the first phase of digestion. Pectin and tannic acid content were reduced by 62.27 and 27.94% respectively in the reactor with anaerobic consortium. Boopathy (1988) reported that cellulose, hemicellulose and protein were reduced by 39.43, 58.2 and 43.81% respectively during anaerobic digestion of coffee pulp waste with digester slurry as inoculum. The same trend was observed in the study with cowdung slurry which recorded 51.25, 15.94, 47.44and 45.85% reduction in pectin, tannin, cellulose and hemicellulose respectively. The anaerobic degradation of gallotannins was first reported by Field and Lettinga (1987) who observed the breakdown of tannic acid by a consortium of anaerobic sludge bacteria. When gallotannins were presented at subtoxic concentration, there was a high conversion of tannins into methane. In the present investigation, the per cent reduction of tannic acid was very less (27.94%) when compared to cellulose and hemicellulose. This might be due to the complex structure of tannins which
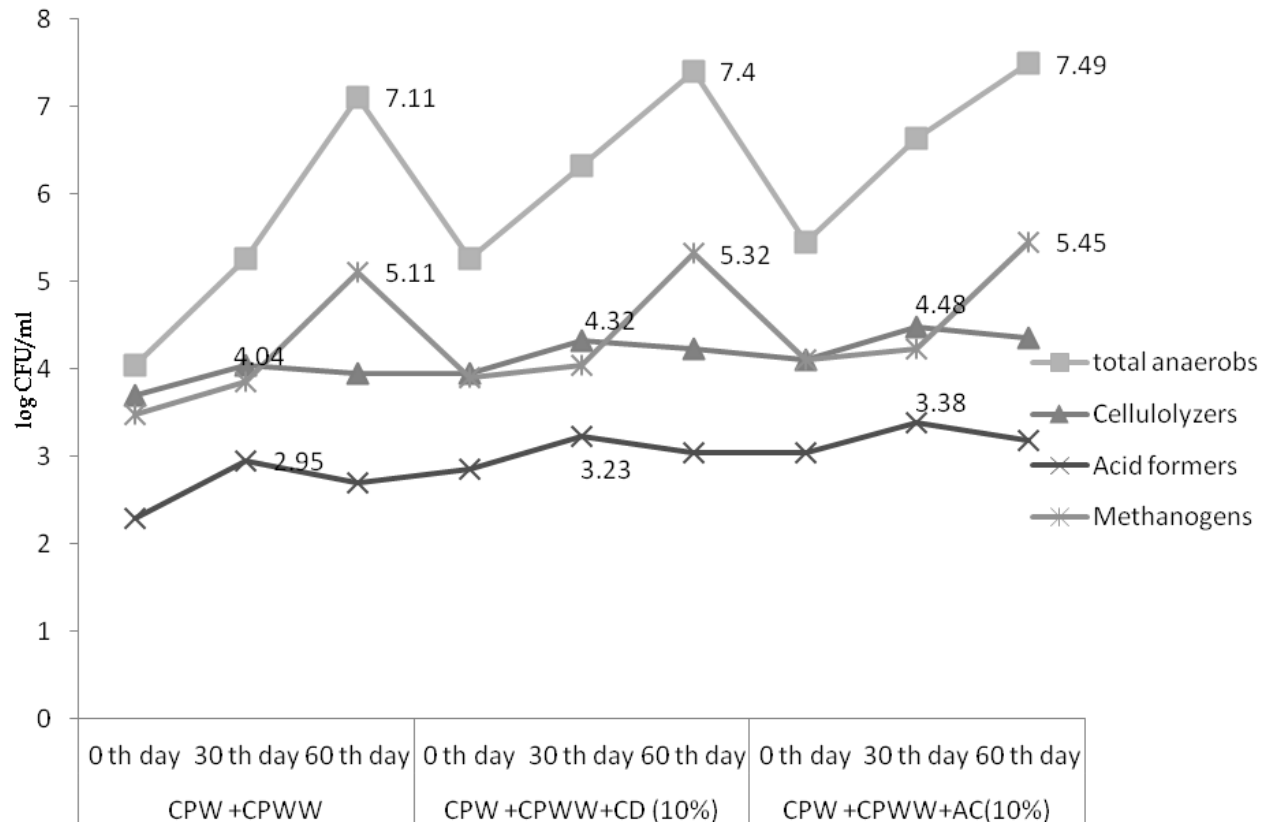
Figure 3. Population dynamics during anaerobic fermentation of the coffee processing wasters.

was not easily hydrolysed. This could be overcome by pre treatment of feed with acid or alkali.

The initial increase of reducing sugars could be attributed due to the hydrolysis of the complex polymers and its subsequent reduction in the later stages is caused by the conversion of reducing sugars to volatile fatty acids and Hydrogen molecules. This confirmed well functioning of the system with succession of microbes.

Methanogenesis and Methane recovery

The average biogas production in reactor with CPW and CPWW with cowdung slurry as inoculums was 4.2 m³ and methane recovery was 62%. In this case also, TS reduction increased with increase in time, but more stability was attained and no noticeable disturbance occurred in the system. From the results its known that the the biogas production prolonged one week more in reactor with cowdung slurry as inoculum than anaerobic consortium but the cumulative yield is less. The advancement in the reactor with anaerobic consortium is due to earlier build up and acclimatization of the ino-culated cells and depletion of nutrients in later stages. From the results, it can be concluded that anaerobic consortium as inoculum performed better than cow dung

slurry as inoculum for anaerobic fermentation of coffee processing wastes (Figure 3).

From the microbial dynamics study during anaerobic digestion, it was inferred that in the first phase of anaerobic fermentation of coffee processing wastes, the population of acid formers and cellulolytic bacteria increased. Similar increase population was observed by Boopathy (1988). The methanogenic population was found to be higher after 30th day and maintained till the end of the experiment.

Maximum methanogenic population was observed in coffee waste digester with anaerobic consortium (10%). This might be due to the presence of viable and active methanogenic organisms with the ability to adapt a new environment. This finding confirms that the developed anaerobic consortium is very good inoculum for initiating microbial loads in the anaerobic fermentor.

Techno-feasibility of anaerobic consortium developed in the study

From the study, it was inferred that approximately 27.2% of expenditure on electricity can be saved. Biogas generated during anaerobic fermentation of 5 ton of coffee pulp waste is equivalent to 544 KW h units of
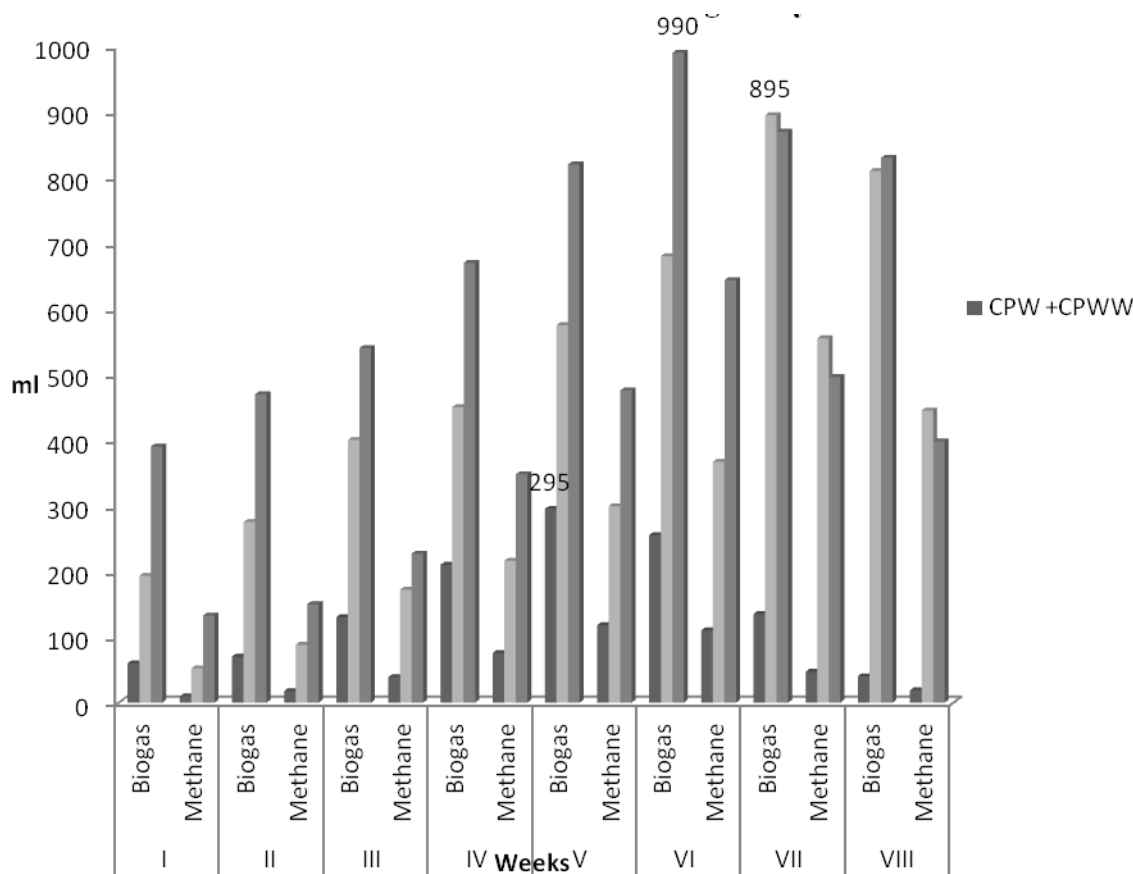
Figure 4. Comparison of biogas and methane production by anaerobic consortia with cowdung slurry.

electricity which can replace 27.2% of total expenditure on electricity. According to previous studies, the potential daily yield of biogas from 2 tonnes of coffee pulp was approximately 131 m^3, equivalent in terms of its fuel value to 100 L of petrol. Boopathy and Mariappan (1984) reported a maximum biogas production of 52 m^3 with coffee waste, cow dung and old slurry in the ratio of 3:1:1.

Conclusion

Among the two inoculum sources, anaerobic consortium and cowdung slurry, the anaerobic consortium developed in the present study showed best results interms of biogas and methane yield with highest consistency of TS removal and pH of the coffee processing wastes. The anaerobic consortium developed could retain metha-nogenic biomass and other anaerobic bacterial load which could be used for treating coffee wastes without any dilution or neutralization. This has provided a new concept of subjecting very high strength coffee process-sing wastes for biomethanation employing anaerobic consortium under high organic loading rate. The post methanation solids obtained can be used as manure and

the effluent still needs to be treated to meet the pollution control standards. The bioenergy can be converted interms of electricity saving 27.2% of power consumption with anaerobic consortium.

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Full Length Research Paper

Soil-transmitted helminth infections among school children in rural communities of Moro Local Government Area, Kwara State, Nigeria

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We investigated stool samples of primary school pupils in four rural communities of Moro Local Government Area of Kwara State for helminthic intestinal parasites and its intensity. Four hundred and thirteen (413) pupils submitted their samples for examination. The samples were examined by wet preparation, formol ether concentration methods and Stoll's technique to estimate the worm burden of positive stool samples. Overall prevalence of soil transmitted helminths (STH) in the four communities was 41.9%. The ova/larvae of STH parasites detected were hookworm, *Ascaris lumbricoides, Trichuris trichiura* and *Strongyloides stercoralis* with prevalence of 15.4, 11.3, 8.1 and 7.1%, respectively. Generally, the intensity of parasitic infections among the pupils was moderate. STH infection was more in 15-20 years age groups than those of 5-9 years and 10-14 years. Risk factors that contributed to high prevalence of STH infections among the pupils and methods of control were discussed.

Key words: Soil-transmitted helminths, parasitic intensity, Moro Local Government Area, school pupils, risk factors.

INTRODUCTION

Soil-transmitted helminths (STH) infections are among the most common infections worldwide and affect the poor and deprived communities. The four main soil-transmitted helminth infections are ascariasis, trichuriasis, hookworm and strongyloidiasis caused by *Ascaris lumbricoides, Trichuris trichiura*, *Ancylostoma duodenale/Nector americanus* (hookworm) and *Strongyloides stercoralis*, respectively. Recent estimate suggests that *A. lumbricoides* infects over one billion people, *T. trichiura* 795 million and hookworm 740 million (WHO, 2013). Generally, more than 1.5 billion people or over 24% of the world's population are infected with these infections worldwide (WHO, 2013). Sub-Saharan Africa has the highest prevalence of these helminth parasites than Latin-America, Caribbean, Middle-east and North Africa (WHO, 2013; Murry and Lopez, 1996; Chan et al., 1994).

Adult hookworm inhabit the upper part of the human small intestine, while *Ascaris lumbricoides* parasitize the entire small intestine and adult *Trichuris trichiura* lives in the large intestine of the human gastrointestinal tract (Bethany et al., 2006).

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STH infections are widely distributed throughout the tropics and subtropics, where climatic conditions are very important determinant in transmission of these infections (Bethany et al., 2006). Adequate moisture and warm temperature are essential for larval development in the soil (de Silva et al., 2003; Brooker and Micheal, 2000). Other important determinants in epidemiology of soil-transmitted helminths are poverty, inadequate water supply, sanitation and poor personal hygiene especially shoe wearing and hand washing (de Silva et al., 2003). Those conditions favour soil-transmitted helminth species and infection commonly occurs as co-infection. STH infections have profound effect on health of growing children, such as chronic ill health and insidious clinical presentation (Brooker and Micheal, 2000). Such children are mal-nourished, have stunted growth, intellectual retardation, anaemia and poor school attendance. Morbidity from these infections and rate of transmission are directly related to the number of worms harboured in the host (Anderson and May, 1991). The intensity of infection is the main epidemiologic index used to determine level of soil-transmitted infection.

Malete, Elemere, Ore-Olumoh and Animaje are small rural communities of Moro Local Government of the central senatorial district of Kwara State. Most of the inhabitants of these villages are farmers, hunters and traders. The water supply is mainly through boreholes, while others depend on locally dug wells, streams/rivers and ponds. Most dug wells dried during dry season (between November and March) then villagers use streams/rivers or ponds as sources of their domestic water supply.

There is continual need to call the attention of the world to the burden of STH infections, some of which are among the neglected tropical diseases. The total burden of diseases due to STH infections and its consequences could be prevented in high-prevalence communities by massive de-worming of school age children (WHO, 2013). Knowledge of the distribution and extent of intestinal helminth infections in rural communities is thus a prerequisite for planning and for effective treatment. There has been no previous report on epidemiology of STH infections in these communities. This survey assessed the prevalence and intensity of soil-transmitted helminth in school age children of rural communities and conditions that might influence their transmission.

MATERIALS AND METHODS

Study site

The study was carried out among the pupils of ages 5-20 years in primary schools at Malete, Elemere, Ore-Olumoh and Animaje rural communities of Moro Local Government area of Kwara State, Nigeria. Kwara State is within the middle belt region of Nigeria and is the link state between the Northern and the Western parts of Nigeria. I t is within the Savannah region and the climate is of equatorial type with rainy and dry seasons. The annual rainfall ranges from 1,000 to 1500 mm. The minimum temperature range is 24-32°C while mean the maximum range is 35-38°C (Kwara State Today, 1997). The communities' water sources were traditionally dug wells, boreholes usually provided by government or its agencies, while nature sources are stream/river and ponds.

Study population

The study population consisted of registered primary school children because they form most accessible age group and most vulnerable to STH infection community. A questionnaire survey data from the pupils included age, type of toilet available, sex, hand washing habit, water sources, weight and occupation of parents or guardians. Ethical approval for the study was granted by the Kwara State University, Malete. Further clearance was obtained from local government health board. Oral consent was obtained from the village heads, clan heads and parents, in addition head and school teachers. Those who refused to give consent were excluded from the study.

Sample collection

All pupils that have consented to the study were enrolled by collection of their survey biodata within the months of February to May, 2013. Children that have received anti-helminthic drugs (randomly and infrequently distributed by State and Local Government Authority) within the past three months in all the schools were excluded from the study. A labelled sterile universal plastic bottle with pupil's name and serial number from the register of biodata was given to each pupil. They were then given sample collection instruction that was done in the local language of the pupils for understanding. These samples were taken to the laboratory as soon as received, usually less than two hours after collection in a carton.

Sample examination

Macroscopic examination was done on each sample as soon as they reached the laboratory. This was used to assess the diarrhoeic condition of the sample. Particular attention was paid to watery, loose or formed stools including presence of mucus and blood in stool. Wet preparation of each sample was done using normal saline and examined immediately.

Ridley modified formol ether concentration technique (Cheesbrough, 1987) was used to improve on the recovery of the ova and larvae of helminth parasites from the samples that were missed in wet preparation. The sediments were examined by placing one drop each on the centre of the slide covered with cover-slip and they were examined, parasites were identified as previously described (WHO, 2004). The eggs and larvae count was done in formol ether concentration technique by weighing 1 g faeces. The entire preparation was examined and eggs found were counted, which gave the number of eggs per gram of faeces. Chi-square test was used to compare the data, P value less than 0.05 was considered significant using SPSS package.

RESULTS

The villages were Malete, Elemere, Ore-Olumoh and Animaje with samples population size of 156 (males 81, female 75); 115 (males 47, females 68); 75 (males 38, females 37) and 67 (males 31, females 36) respectively.

Table 1. Prevalence of intestinal STH infection in rural communities of Moro LGA.

Organism	Male (%)	Female (%)	Total No (%)
A. lumbricoides	19 (4.7)	27 (6.6)	46 (11.3)
Hookworm	36 (8.8)	27 (6.6)	63 (15.4)
T. trichiura	17 (4.2)	16 (3.9)	33 (8.1)
S. stercoralis	12 (2.9)	17 (4.2)	29 (7.1)
Overall Prevalence	84 (20.6)	87 (21.3)	171 (41.9)

Excluded pupils due to use of drug and non-consent were less than 10 pupils from each village.

In these rural areas, we detected intestinal helminths in 171 (41.9%) of 413 specimens, distributed as follows: Malete 115 (73.7%), Elemere 86 (74.8%), Ore-olumoh 70 (92.1%) and Animaje 50 (74.6%). The overall distribution of the STH parasites among the pupils of the rural communities is shown in Table 1.

Hookworm parasite (15.3%) was the most frequently detected STH parasites, while *S. stercoralis* (7.1%) occurred least. Overall sex distribution showed that female pupils (21.3%) were slightly more infected with STH parasites than the male pupils (20.6%).

Table 2 shows that there were 197 (47.7%) male pupils that registered for this study, 84 (42.6%) of which had at least one helminth parasite in their stool sample, while out of 216 (52.3%) of the female pupils registered for the study, 87 (40.3%) had at least one parasite detected.

The pupils were grouped into three categories based on their age: 5-9 years (43.3%), 10-14 years (52.5%) and 15-20 years (4.1%). Age group 15-20 years had the higher prevalence of helminth parasite of 47.1% than age groups five to nine years (41.9%) and 10-14 years (39.6%). There were fewer pupils in age group 15-20 years because most of their mates have gone to high school or gone to learn trade or apprenticeship.

The education system is divided into first basic primary school, that is, Primary 1-3 with 213 (51.6%) pupils and second basic primary school that is, primary 4-6 with 200 (48.4%) pupils. Pupils in first basic primary school had a higher prevalence of 42.7% of helminth infection than 40.0% of second basic primary school. Age distribution is presented in Table 2.

There were three main sources of domestic water supply to these communities where the pupils lived: bore hole, locally dogged well and stream/river. Parasites were found at higher frequency in pupils that use well (54.1%), than river/stream, 47.1% and bore hole, 38.6%. However, the difference was not significant at P > 0.05.

Most homes in these communities do not have toilet facilities. Nine out of 413 pupils (6.3%) use private latrine while 293 (70.1%) use open-field as places of defaecation while other use public latrine that were built by government or its agents. Higher prevalence of STH parasites were found among pupils that use opened field

(45.7%) than 27.0% and 22% for public and private latrine respectively, this difference was significant at P< 0.05.

Hand washing habit of the pupils was also investigated in this study with three options: hand washing before meals 177 (42.9%), hand washing after use of toilet 109 (26.4%) and those that neither washes hands before meals nor after use of latrine 127 (30.8%). The prevalence of helminth parasites detected was 13.8, 12.5 and 14.0% in those that wash hands before meals, those that wash hands after use of latrine and those that neither wash before meals nor after use of latrine, respectively.

In this study, four helminth parasites were detected; however majority (30.8%) of them occurred as single parasitic infection in pupils. While very few had multiple infections. Only 19 (4.6%) had two parasites in their stool while only 2 (0.5%) had three different parasites in their stool.

Worm load is very important in determination of infection outcome by helminth parasite. There were five categories per preparation: scanty (1-3), few (4-10), moderate (11-20), many (21-40) and very many (over 40) per a gram of stool (Cheesbrough, 2002). In this present study, most of the pupils (40.1%) had moderate infection, while pupils with heavy infection had prevalence of 8.8%.

DISCUSSION

Previous studies on estimated worm burden showed that globally, 39 million disability adjusted life years were lost due to intestinal helminthiasis (WHO, 1990; Chan, 1997). This modal also estimated that about 70% of the total worm burden of diseases is due to STH infections. High prevalence of STH infection was found among school age pupils of rural communities in Moro LGA of Kwara State. Male pupils had a higher prevalence (42.6%) than the female (40.3%). The present study revealed a high prevalence of 41.9% of STH infection among the pupils in rural communities of Moro Local Government Area. Each of the four rural communities studied had a varying prevalence as illustrated in Table 1. Eggs of hookworm (*A. duodenale*/*N. americanus*) had the higher prevalence than *A. lumbricoides* and whipworm *T. trichiura*. This prevalence was similar to previous studies in several

Table 2. Risk factors and intestinal helminth infections among school children in rural communities of Moro LGA.

Index	Soil transmitted helminthic parasites				
	Yes (%)	No (%)	Total (%)	X^2	P-value
Sex					
Male	84 (42.6)	113 (57.4)	197 (100)	0.2369	0.6264
Female	87 (40.3)	129 (59.7)	216 (100)		
Age group (years)					
5-9	75 (41.9)	104 (58.1)	179 (43.3)		
10-14	86 (39.6)	131 (60.4)	217 (52.5)	04851	0.7846
15-20	08 (47.1)	09 (52.9)	17 (4.1)		
Education level (Primary 1-6)					
1-3	91 (42.7)	122 (57.3)	213 (51.6)		
4-6	80 (40.0)	120 (60.0)	200 (48.4)	0.3152	0.5744
Water Source					
Bore hole	124 (38.6)	197 (61.4)	321 (77.8)		
Well	13 (54.2)	11 (45.8)	24 (5.8)	3.498	0.1738
River/stream	32 (47.1)	36 (52.9)	68 (16.5)		
Latrine facility availability					
Private	2 (22.2)	7 (77.8)	9 (2.2)		
Public	30 (27.0)	81 (73.0)	111 (26.9)	12.956	0.0014
Openfield	134 (45.7)	159 (54.3)	293 (70.1)		
Hand washing habits					
Handwashing before meals	57 (32.2)	120 (67.8)	177 (42.9)		
Handwashing after toilet use	52 (47.7)	57 (52.3)	109 (26.4)	8.817	0.012
Neither any of the above	58 (45.7)	69 (54.3)	127 (30.8)		

developing tropical countries (Tadesse, 2005; Ali et al., 2003; Agi, 1995; Aghere et al., 1995). The average prevalence in some West African countries (Nigeria, Togo and Guinea Bissau) was 5 to 60%) (Molbak et al., 1994).

These three nematodes have been implicated in growth retardation in children in tropical countries (Stephenson et al., 1989), for various reasons such as the cause of iron deficiency anaemia, malnutrition, malabsorption and vitamin A deficiency (Roche and Benito, 1999). In addition, some of the pupils had multiple infections of these parasites. Result showed that 4.6% of the pupils had two STH co-infections and 0.5% had three STH parasites. Multiple infections would have an over-whelming side effect on health of the pupils concerned. Previous report indicated that soil transmitted helminth species are commonly co-endemic (de Silva et al., 2003). Rhabidiform larvae of *Strongyloides stercoralis* were also detected in the faecal samples with low prevalence of

7.1%. Female pupils had higher prevalence of 4.2% while the male pupils had 2.9%. This parasite has also been implicated in growth retardation and stomach pains in pupils.

Other intestinal helminths such as *Enterobius vermicularis* and *Hymenolepis nana* detected in previous studies by Jarallah (2012) and Tappe et al. (2011) in Iraq and Iran, respectively, were not detected in our study, though they were searched for. Age distribution showed that STH infections were more prevalence among age group 15-20 years (47.1%). This is because at such age pupils indicate changes in exposure to conditions that favour the transmission of the infective stage of the STH parasites. Other risk factors to acquisition of STH parasites in rural areas included the use of open-field for toilet as in 70.1% of the pupils studied. About 2.2% of the pupils came from homes that have private latrine facilities. Hand-washing habit of the pupils in these communities is very poor. Only 42.9% of the pupils wash

Table 3. Parasites and intensity.

Parasite in stool sample	Parasites co-infections			
	1	2	3	Total
No. (%)	127 (30.8)	19 (4.6)	2 (0.5)	148 (35.8)

Parasite intensity

Organism	Scanty (+)	Few +	Moderate ++	Many +++	Very many ++++	Total
Hookworm	6	10	33	6	8	63
A. lumbricoides	8	7	20	8	3	46
T. trichiura	11	6	10	4	2	33
S. stercoralis	6	10	6	5	2	29
Total	31 (18.1)	33 (19.3)	69 (40.1)	23 (13.5)	15 (8.8)	171 (100)

their hands before meals, and 26.4% wash their hands after use of toilets while 30.8% of the pupils do not wash hands after the use of toilets nor before meals. The personnel hygiene and poor socio-economic factors contributed to the high prevalence of STH infections in this study (Adedoyin et al., 1990; Allen et al., 2004).

Other important determinants in transmission of STH parasites are poverty and inadequate water supplies and sanitation (Bethany et al., 2006). In the present study, 77.8% of the pupils use bore holes as source of domestic water supply and had least prevalence of 38.6% of STH infection while those that use well had prevalence of 54.2% and stream/river was 47.1% prevalence of STH infection.

Intensity of infection is the main epidemiological index used to describe STH infection, this intensity is measured by the number of eggs per gram of faeces. The distribution of these eggs/larvae showed that majority of the pupils had moderately (++) infection while few were in the two extremes of scanty (+) and very many (++++) (Table 3). Generally, only STH infections of moderate and high produce clinical manifestation (Chan et al., 1994). Previous study indicated that the highest intensity of infections is most common among the school age pupils than adults (Chan et al., 1994; Brooker et al., 2006), and that morbidity from these infections are directly related to the numbers of worms harboured in the host (Arderson and May, 1991).

In conclusion, we reported high prevalence of STH infections among the school pupils in rural communities of Moro LGA of Kwara State and we discussed factors that contributed to this high prevalence. All infected pupils were treated and each class was given health education talk with emphases on hand-washing, wearing of shoe/sandal and personal hygiene. We recommend an improved health education and supervision, regular de-worming, provision of toilets at homes and schools and improved sanitary disposal of human wastes among the pupils and others in the communities to reduce trans-mission of STH infections.

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Full Length Research Paper

Microbacterium arborescens AGSB sp. nov., isolated from the rhizosphere of sand dune plant, *Ipomoea pes caprae*

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Phenotypic and phylogenetic studies were performed for the facultative alkalophile from the rhizosphere of *Ipomoea pes caprae*, a plant growing on coastal sand dunes. The isolate was Gram positive and showed optimum growth at pH 10.5. Chemotaxonomic analysis revealed that the isolate contained type B1 peptidoglycans with L-lysine as the diamino acid; rhamnose and galactose were the cell wall sugars and belonged unambiguously to the genus *Microbacterium*. The major menaquinones were MK-11 and MK-12. The 16S rDNA sequence of the *Microbacterium arborescens* isolate was deposited in the GenBank with an accession number DQ287961. The phylogenetic and phenotypic distinctiveness of the strain indicates it as a novel *Microbacterium sp.,* named as *M. arborescens* AGSB.

Key words: facultative alkaliphile, *Microbacterium arborescens AGSB*, Coastal sand dune vegetation, *Ipomoea pes caprae*, 16S rRNA sequencing.

INTRODUCTION

The sand dune ecosystem is a stressed habitat with only certain type of vegetation surviving in this ecosystem, one such plant is *Ipomoea pes caprae* which is commonly found on coastal dunes. Although a nutrient limiting ecosystem, in the rhizosphere, plant litter contributes to humus and organic matter on which the microbiological communities survive. Few studies on the bacterial species present in coastal dunes have been published. The isolates obtained in this study have been identified as *Acinetobacter, Pseudomonas, Paenibacillus, Microbacterium, Agrobacterium Chryseobacterium* and *Pseudomonas* (Park et al., 2005, 2006; Leveau et al., 2009; Godinho and Bhosle, 2010; Muthezhilan et al., 2012; Gaonkar et al., 2012). Earlier studies on isolates from this ecosystem have shown their ability to produce exopolymers which aid in sand aggregation and stabilize the dunes (Godinho and Bhosle, 2009). A potent exopolymer producing isolate was selected for identifying it to genus level. We report here the characteristics of a predominant facultative alkaliphile from the rhizosphere of *Ipomoea pes caprae* that exhibited orange, pigmented colonies and gram positive, non sporing regular rods which has previously not been reported. Further, the isolate was identified using polyphasic taxonomic tools including chemotaxonomic and 16S rRNA sequencing. Based on the results, a new species is proposed as *Microbacterium arborescens* AGSB.

MATERIALS AND METHODS

Strain, cultivation and maintenance

The strain chosen for this study was a predominant isolate from coastal sand dunes; it was isolated from the rhizosphere of *Ipomoea pes caprae* sand dune vegetation by serial dilution method of the rhizosphere sand and then plating on polypeptone yeast extract glucose agar (PPYG) medium, pH 10.5. The plates were incubated for 2 days at 30°C on polypeptone yeast extract glucose agar (PPYG) medium containing (g/l): peptone, 5; yeast extract, 1.5;

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Figure 1. Scanning Electron Micrograph of *Microbacterium arborescens* AGSB.

disodium hydrogen phosphate, 1.5; sodium chloride, 1.5; magnesium chloride,0.1; agar, 15, glucose (10%); sodium carbonate (10%); pH 10.5 (Horikoshi, 1987). Biomass for chemotaxonomic analysis was obtained by growing the strain aerobically in PPYG without agar (Polypeptone yeast extract glucose broth) on an orbital shaker at approximately 160 rpm for 2 days before harvesting by centrifugation (10,000 rpm for 20 min).The cells were washed twice with sterile distilled water and freeze dried.

Morphological, physiological and biochemical characterization

Cell morphology was determined by phase contrast microscopy and electron microscopy (Figure 1), motility by the hanging drop method, biochemical characteristics were determined by the method described (Takeuchi and Yokota, 1994; Zhang et al., 2010; Shivakumar, 2012).

Chemotaxonomic methods

Preparation of cell walls and determination of peptidoglycan structure was carried out by the methods described by Schleifer and Kandler (1972). Menaquinones were extracted and analysed as described by Komagata and Suzuki (1987), Collins (1985) and Minnikin and Goodfellow (1985). Polar lipids were extracted and analyzed by Thin Layer Chromatography according to Komagata and Suzuki (1987).

DNA extraction, PCR amplification and sequencing

A single isolated colony of the selected bacterial cultures was taken from agar plate and suspended in 50 µl of colony lysis solution. The reaction mixture was incubated at 55°C for 15 min followed by proteinase K inactivation at 80°C for 10 min. The reaction mixture was centrifuged at 15,000 rpm at 4°C for 15 min. The supernatant containing genomic DNA was directly used as template in PCR reaction. PCR amplification of almost full length 16S rRNA gene was carried out with eubacteria specific primer set 16F27N and16R1525XP, in a 25 µl final reaction volume, containing about 10 ng of genomic DNA, 1X reaction buffer, 0.4 mM (each) deoxynucleoside triphosphates (Invitrogen), 0.5 U of DNA Polymerase (New England Labs, UK) and the final volume was made 25 µl by adding sterile nuclease free water. The PCR was performed in an Automated Gene Amp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, USA) under the following conditions. The amplification conditions were as follows: 94°C for 1 min (denaturation), 55°C for 1 min (annealing), 72°C for 1.30 min (elongation), and 72°C for 10 min final elongation. Expected PCR product of around 1.5 kb was checked by electrophoresis of 5 µl of the PCR product on 1% agarose gel in 1X TBE buffer and stained with ethidium bromide (0.5 µg/ml). The PCR product was precipitated by PEG-NaCl (20%PEG in 2.5MNaCl) precipitation at 37°C for 30 min. The reaction mixture was centrifuged at 12,000 rpm for 30 min at room temperature. The supernatant was discarded and the pellet was washed twice with 70% ethanol. After drying the pellet, it was resuspended in 5 µl of sterile nuclease free water. One microliter (50 ng) of purified 16S rRNA PCR product was sequenced by 16S rRNA specific primer that is 16F27N, 530F and 16R1525XP.

Phylogenetic analysis

Purified double stranded PCR fragments were directly sequenced using BIG DYE Terminator cycle sequencing ready reaction kit (v3.1) in ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, USA). The sequences were compared with the 16S rDNA sequences available in the public databases from a BLAST search, and identified to the generic level.

Table 1. Significant characteristics of the isolate *Microbacterium arborescens AGSB.*

Characteristic	*M. arborescens AGSB*
Colour of the colony	Orange
Motility	Positive
Hydrolysis	
Gelatin	Positive
Starch	Negative
H_2S production	Positive
VP test	Negative
Arginine dihydrolase	Negative
Assimilation	
Arabinose	Positive
N-acetyglucosamine	Positive
Malate	Positive
Citrate	Positive
Phenyl acetate	Negative
Fumarate	Positive
Propionate	Negative
Acid from	
Glucose	Positive
Cell wall diamino acid	Lysine
Major menaquinone acid	MK-11,12

The 16S rDNA sequences were aligned using CLUSTALX (ftp://ftp.ebi.ac.uk/pub/software/clustalw2) with *Microbacterium* nucleotide sequences derived from GenBank. The trees were constructed using the neighbour-joining method. The PHYLIP package (Felsenstein, 1993) was used to generate trees with the four algorithms and the trees were viewed using the TREEVIEW package (Page, 1996; Felsenstein, 1981, 1985). Tree topologies were evaluated by bootstrap analysis of the neighbour-joining tree using the original dataset and 1000 bootstrap datasets.

RESULTS AND DISCUSSION

The phenotypic characteristics of the strain are in agreement with the placement of the strain in the genus *Microbacterium.* The strain showed biochemical characteristics as described in Table 1. The specific characteristics which indicated that the isolate belongs to the genus *Microbacterium* are hydrolysis of gelatin, hydrogen sulphide production, assimilation of malate, citrate, n-acetylglucosamine, fumarate and arabinose. Chemotaxonomic analysis of the isolate revealed the presence of the amino acid lysine and sugars rhamnose and galactose in the cell wall, unsaturated menaquinones MK-11 and MK-12 while polar lipids present were diphosphatidylglycerol and phoshatidylinositol. Based on these results and the identification in Bergey's Manual of Systematic Bacteriology, this isolate has been designated to genus *Microbacterium.* The present isolate is a facultative alkaliphile and is present in highly stressed

environment. There have been no reports so far of a facultative alkaliphile from coastal sand dune ecosystem.

In the present study, an attempt was made to sequence the 16S rDNA in order to study its relationship to other species of *Microbacterium*. The phylogenetic analysis of the 16S rDNA sequence was accompanied by PCR amplification of approximately 1500 base pairs using universal primers. The resulting PCR segments were sequenced and optimally aligned and phylogenes were constructed using alogorithms available in the PHYLIP site of phylogenetic analysis programs. Phylogenetic tree was constructed by neighbour joining method (bootstrap method) as shown in Figure 2. The sequences were deposited in the GenBank and the extent of similarities with other *Microbacterium* strain were determined. The phylogenetic tree showed the strain to be 97% similar to *Microbacterium* sp.MSCB7. The genus *Microbacterium* was established to accommodate a diverse collection of gram positive non spore forming rods isolated during studies on lactic acid producing bacteria. Extensive phylogenetic studies have recently resulted in amalgamation of the genera *Microbacterium* and *Aureobacterium* into a redefined genus *Microbacterium* (Takeuchi and Hatano, 1998)

The isolate characterized in the study is a bacteria which can tolerate a high alkaline pH and survive in stressed conditions where the moisture holding capacity of the sand is minimal. Interestingly, this isolate was found to produce large quantities of exopolysaccharide
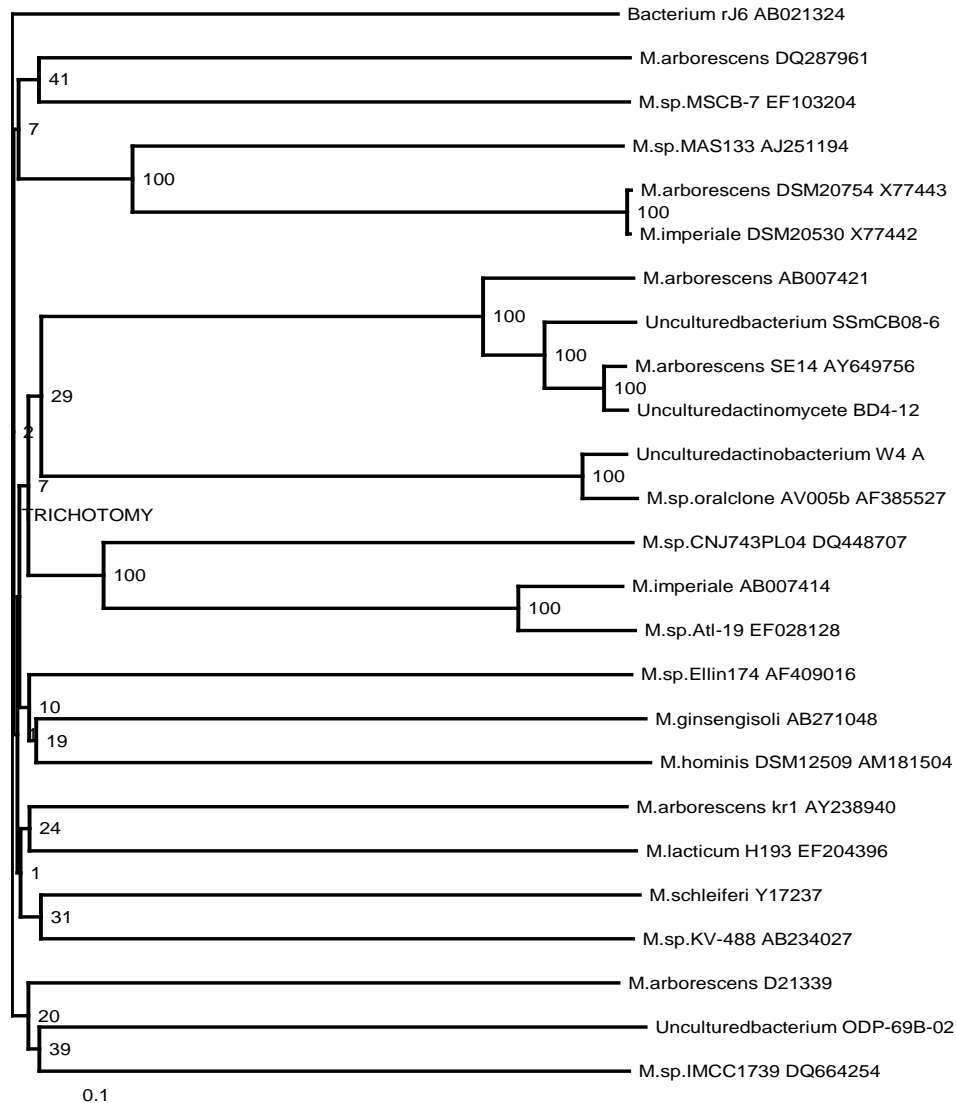
Figure 2. Unrooted tree showing the phylogenetic relationships of *Microbacterium arborescens* sp. nov. and members of the genus *Microbacterium* based on 16S rDNA sequences. The tree, constructed using the neighbour-joining method, boot strap values, expressed as percentage of 1000 replications, are given at the branching points.

(Godinho and Bhosle, 2009) which perhaps supports its adherence and survival in this otherwise stressed eco-system. Based on these results, we report the isolation and characterization of a new facultative alkaliphile *M. arborescens* AGSB.

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Full Length Research Paper

Characterization and propensity of white spot syndrome virus extracted from imported specific pathogen free (SPF) pacific *Litopenaeus vannamei* brooders progeny by performing SF9 cell line culture

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Shrimp viral diseases have caused severe production and economic losses in the past two decades. A complete understanding of shrimp viruses is dependent upon the development of laboratory techniques for the maintenance and culturing of these viruses and host cells. This investigation was done to characterize the cell line culture from specific pathogen free *Litopenaeus vannamei* and its susceptibility to revise the cytopathic effects of white spot syndrome virus. A cell culture was successfully developed from insect cell SF9. Cytopathic changes like enlarged cells, focal lesions, shrunken and clumped cells were observed in white spot syndrome viruses (WSSV) infected SF9 cell cultures from 24 to 120 h duration. In the present study, conditions for the successful primary culture of insect SF9 cells for WSSV infection have been established. The conformation of WSSV infection in SF9 cell line was by polymerase chain reaction using target gene of WSSV419 like proteins and followed by electrophoresis. The WSSV result was positively as 550 bp in SF9 cell line and in the control sample. This is the first report on the development of primary cell culture of WSSV, host species of *L. vannamei* using insect cell line SF9.

Key words: White spot syndrome viruses (WSSV), SPF, *L. vannamei,* SF9 cell line, cytopathic effect, pathogen, economy

INTRODUCTION

Crustacean cell culture has gained momentum due to viral diseases affecting commercially important species. Hence, cell culture techniques were developed: (a) to assist in understanding the mechanism of host pathogennesis interaction (Chen et al., 1989), (b) to produce large amount of viral material for their characterization and (c) to improve tools for diagnosis and cure of diseases. Attempts have been made to establish several cell culture systems of shrimps (Al-Mohanna and Nott 1987; Chen et al., 1986, 1995; Ke et al., 1990; Nadala et al., 1993; Hsu et al., 1995; Toullec et al., 1996; Mulford and Austin, 1998; Mulford et al., 2001; Uma et al., 2002) and other crustaceans (Peponnet and Quiol, 1971). Yet, for reasons that remain obscure, all endeavors to develop cell cultures from marine invertebrates have been ineffective so far (Rinkevich et al., 1994; Bayne, 1998; Rinkevich, 1999; Mothersill and Austin, 2000) despite the acknowledged need for cell cultures from species that are important in aquaculture or in the pharmaceutical industry (Rinkevich et al., 2005).

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Attempts were made by various researchers in establishing the primary cell culture and continuous cell lines from different organ sources of shrimp (Luedman and Lightner, 1992; Purushothaman et al., 1998; Roper et al., 2001). Successful attempts on the development of primary cell culture derived from hepatopancreas are few (Toullec et al., 1996). Recently, the successful development of primary shrimp cell cultures has been reported from many laboratories (Chen et al., 1986, Hsu et al., 1995; Luedeman and Lightner, 1992; Nadala et al., 1993; Toulled et al., 1996). Lu et al. (1995b) and Tapay et al. (1997) developed an *in vitro* quantal assay for yellow head virus (YHV) and China Baculo-like virus (CBV) using primary lymphoid cells of the white shrimp *Penaeus vannamei* and *Penaeus stylirostris*.

With the rapid expansion of high density aquaculture of penaeid shrimp, the detection of infectious diseases, especially of viral etiology, has become increasingly important. Several viruses including *Baculovirus penaei* (BP) (Couch, 1974; Lightner, 1983), *Penaeus monodon*-type baculovirus (MBV) (Lightner and Redman, 1981; Lightner, et al., 1983a) baculoviral midgut gland necrosis virus (BMNV) (Sano, et al., 1981) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Lightner, et al., 1983b), white spot disease (Inouye, et al., 1994; Momoyama et al., 1994; Nakano, et al., 1994) and yellow head disease (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993) are known to be the causative agents of disease and mass mortality of cultured penaeid shrimp. Although, several papers have been published on these viral diseases of cultured shrimp, almost all of the studies were limited to histopathology observations using light and electron microscopy.

Presently, an effort has been made to characterize the white spot syndrome viruses (WSSV) post infected tissues of lymphoid organ, gill and mid-gut gland using insect cell line namely SF9 of the offspring's of SPF *L.vannamei* brooders originating from Thailand.

MATERIALS AND METHODS

Sample collection and processing

A total of 20 individuals with ABW 5 - 7g SPF *L.vannamei* offspring's of SPF brooders with ABW 30 -60 g were randomly collected from prime division of South Indian grow out ponds. Suspected WSSV infected insect cells enlightening a hypertrophic appearance were obtained from moribund shrimps. WSSV infection in shrimp was confirmed by polymerase chain reaction (PCR) using PCR primers for target genes such as WSSV419 like protein which is similar to VP28 and their amplification size and by specific clinical symptoms like the presence of white spots, lethargy and reddish discoloration of the body.

Following the confirmation of WSSV infection, the non-viral infected SF9 cell cultures were discarded. All the cultures were observed with an Olympus IM inverted microscope for the formation of confluence cell sheet and the presence of cytopathic effect (CPE). The cells with CPE foci were collected and then prepared for analysis as described by Bioserve Biotechnology, India (P) Ltd. Prior to the experiment, all shrimps were sterilized as described

by Chen et al. (1986) using 5% sodium hypochlorite. Subsequently, tissues including lymphoid tissue, 'Oka organ', located at the anterioventral surface of hepatopancreas (Oka, 1969), heart and hepatopancreas were removed and rinsed in double strength (2X) Leibovitz's L-15 medium for 3-4 times.

Growth and maintenance of SF9 cells

Spodoptera frugiperda (SF9) insect cells grown in suspension, serum-free medium (SFM) was used in our experiments for the culture of WSSV cells. The animals were anaesthetized in cold water at 4°C for 10 min and the body surface was sterilized with 2% tincture and 75% ethanol as described by Ke et al. (1990). Flasks: Sterile, disposable 50, 125 and 500 ml flasks were used for culturing the cells. For small culture volumes (10 ml), Nalgene polycarbonate shaker flasks were used. Before use, the flasks were rinsed several times in MQ water and allowed to air dry. Sterilization was done for 15 minutes at 121°C, and air dried for 15 min.

Media preparation for SF9 cells growth

Grace's insect medium (Gibco) was supplemented with 1x with Pluronic F-68, 10% (100 x), (Invitrogen). 5 ml of PenStrep (antibiotics) was added to 500 ml medium and final PenStrep concentration was maintained as 1% for every 50 ml conical flask and 45 ml of Grace medium containing 1 % PenStrep with 5 ml FBS (fetal bovine serum) 0.5 ml Pluronic F-68, 10% (100 x) (PA) was prepared and maintained in four conical flasks.

Thawing and adding of cryogenic cells

The cells that have been frozen in DMSO at a density of 10 mill.cells/ml from a cell culture that is only a couple of days old was used. Two tubes, containing 1 ml of cells (10 mill. cells/ml), out of the liquid nitrogen freezer was thawed in a 26°C water bath. For every 1 ml aliquot of cells, resuspention in 5 ml suspension medium (+PA); 20 mill.cells/ml, 10 ml suspension medium (+PA) was used (15 ml/conical approx).

By centrifuging for 7 min at 1000 rpm the supernatant was decanted. The resuspended cells in desired amount of suspension medium was 30 ml of suspension medium for 20 mill cells /ml; expected confluence is 0.667 mill cells/ml, initially, when cells grew at low density (0.5-1 mill. cells/ml). Cell suspensions were transferred into 50 ml spinner flask.

Determining cell density and viability

200 µl cell suspensions were withdrawn and the cell count was taken. 20 µl cell suspensions were mixed with 20 µl trypan blue which was sucked by capillary forces into the flow chamber. In 10x magnification, the cells were counted in 5 x 4 grids. Dead cells appeared as brown spots, whereas healthy cells had a bright ring. Actual number of cells were counted in grids sized as 25 x 2 (correction for dilution in dye) x factor 10^4 = 0.5 mill. cells /ml.

Freezing cells and procedure

Freezed cells were counted under the conditions which are at a density of ≥1 x 107 viable cells/ml. By using a freezing medium composed of 50% fresh growth medium and 50% conditioned growth medium (day 2 to 4 cell conditioned media collected from SF9 cultures during subculture procedure) and DMSO to a final concentration of 7.5%, freezing medium was prepared instantly, filter-sterilized before the experiment and chilled at 4°C until used.

Desired quantity of Sf9 cells in spinner flasks were harvested when the cells are in mid-log exponential growth and have a viability of >90%. The viable and total cell counts were calculated to the volume of freezing medium required to yield a final cell density of ≥1x107 viable cells/ml. The cells from cell suspension were centrifuged at 100 x*g* for 5 to 10 min. Supernatant was decanted and the cell pellets were resuspended in the pre-determined volume of chilled freezing medium. Dispensed aliquots of this suspension (frequently mixing to maintained a homogeneous cell suspension) into cryovials and the freezing rate should be decrease to 1°C per minute. The vials were transferred to liquid nitrogen (vapor phase) storage.

In vitro multiplication of WSSV from *L. vannamei* using SF9 cell lines

SF9 cell lines were examined for their ability to propagate WSSV. Viral suspension was prepared as described by Boonyaratpalin et al. (1993) with slight modifications. Briefly, 10% (w/v) gill tissue of WSSV infected *L.vannamei was* homogenized in 2X L- 15 medium and filtered through a 0.2 μm sterile membrane. The virus suspension was then diluted 100 times in 2X L-15 medium and 10 μl of the diluted suspension was inoculated into freshly grown SF9 cell cultures. Control wells were inoculated with an extract of normal gill tissue prepared in the same manner. The inoculated plates were incubated at 28°C and observed daily for cytopathic effect (CPE).

Concentrating the virus

To produce viral multiplicity of infection (MOI) (>10.0), this protocol is used to concentrate the virus from growth medium. The supernatant must be harvested from a non-lytic, serum-free culture. About 33 ml of virus stock were loaded into each of the six 38-ml polyallomer ultracentrifuge tubes. The virus stock was added with 3 ml of sucrose solution per tube and centrifuged at 80,000g for 75 min at 4°C. By decanting the supernatant, relatively pure viral pellet can be visualized which is translucence white, with faint blue colour near the edges. Less pure pellets displayed increased opaqueness and size; their colour ranges from pale yellow to light brown as contamination increases. The pellets were resuspend in 0.5 to 5 ml D-PBS and the cells were further allowed to disrupt completely. The cells were then filtered through a 0.2-μm filter and stored at 4°C.

Viral susceptibility and storage

White spot syndrome viruses (WSSV) were used to test viral susceptibility of the SF9 cell line. Eighty percent confluent monolayers of SF9 cells were infected with WSSV at a MOI of approximately 0.1. Cytopathic effects (CPE) caused by the viruses were observed daily using an inverted light microscope. For virus titration, 0.1 ml of 10-fold serial dilutions of virus was inoculated into four wells of sub confluent cells in a 24-well plate (NUNC^{TM} Brand Products). The cultures were incubated for seven days at 27°C, and CPE was recorded daily. The virus titres were expressed as the 50% tissue culture infective dose (TCID$_{50}$) according to the method of Reed and Muench (1938).

Virions are quite stable in standard serum-supplemented growth media. They maintained their integrity and infectious competency for days at elevated temperatures, weeks at room temperature, and months to years at 4°C. The virions were stored for longer than 3 months under serum-free conditions by adding 0.1 to 1% BSA to stabilize the virus. The virus stocks were stored in polypropylene containers to prevent nonspecific binding of virus. They were retitered periodically if used as inoculates. Loss in virus titter were minimal (<10%) with this protocol. Virus-containing supernatant was transferred to a sterile, capped centrifuge tube and centrifuged for 5 min at 500 g and decanted. The supernatant was then filtered using a 0.2-μm, low-protein binding filter. The sterile-filtered supernatant was stored in cryotubes and the virus stocks were stored at 4°C and stored in dark conditions avoiding the exposure of light.

Viral DNA extraction and PCR analysis from infected and control cell lines

Genomic DNA extraction was done using DNA isolation kit from Bioserve Biotechnologies India Private Limited for the infected samples and control ones. Briefly, the procedure is outlined as viral replicative DNA intermediates were isolated from whole cell lysates. Cells recovered after trypsinization and one wash were lysed for 16 h at 37°C in lysis buffer (10 mM Tris•HCl, pH 7.4/0.5% SDS/10 mM EDTA, pH 7.4/10 mM NaCl) supplemented with proteinase K (200 μg/ml). The covalently closed circular DNA form was selectively extracted from cells recovered by trypsinization. Cells were lysed at room temperature in a lysis buffer not supplemented with proteinase K. In both cases, cellular DNA was precipitated overnight at 4°C with 1 M NaCl. Nucleic acids were extracted after an overnight lysis at 37°C in lysis buffer supplemented with tRNA (40 μg/ml) and proteinase K (200 μg/ml). DNA was extracted by phenol chloroform and precipitated by isopropanol. Nucleic acids were analyzed by gel electrophoresis procedure on a 1.5% agarose gel containing ethidium bromide (1 μg ml^{-1}). The DNA bands were visualized under ultraviolet transilluminator (Gel Doc 2000, Bio-Rad Laboratories,USA).

For PCR analysis, a 1.0 μl of sample DNA (approximately 100 ng/μl) was added to PCR mixture containing 100 mM Tris HCl (pH 8.3), 500 mM KCl (pH 8.3),2.0 μl MgCl$_2$ (25 mM), 2.0 μl dNTP's (2.5 mM), 1.0 μl primer forward and reverse (each of 10 pm/μl) and 1 u/μl of Taq Polymerase. The PCR was conducted in Gene Amp PCR System 9700 (Applied Biosystem, USA). The amplification condition for WSSV was one cycle of 94°C for 5 min then 38 cycles of 94°C for 30 s , 55°C for 30 s, 68°C for 45 s and 68°C for 7 min. The PCR products (6 - 10 μl) were separated by electrophoresis in 2% agarose gels containing ethidium bromide (1 μg ml^{-1}). The DNA bands were visualized under ultraviolet transilluminator (Gel Doc 2000, Bio-Rad Laboratories,USA).

RESULTS

The cell culture was successfully developed from insect cell SF9 in serum-free medium (SFM). After seeding the SF9 cells, the cells showed attachment to the surface in 24 h. Although, more number of unattached floating cells could be observed initially, a monolayer could be observed by 72 h post seeding. The cells were observed to be spherical in shape. The primary cell culture could be maintained for 12 weeks with seven passages without any undesired effect on the cells. The cytopathic effects was observed in the primary cell culture infected by WSSV. Clumping of cells in a circular fashion and focal lesions of CPE could be observed in 24 h as shown in Figure 1. At 48 h, the cells appeared shrunken in places that showed clumping in 24 h (Figure 2). At 72 h, detachment of the cell clumps and appearance of circular clear areas without cells due to cell lysis occurred (Figure 3). At 96 h, clear circular areas increased in number (Figure 4). Complete "peeling off" cell layer from the culture vessel and scattered abnormal enlarged cells
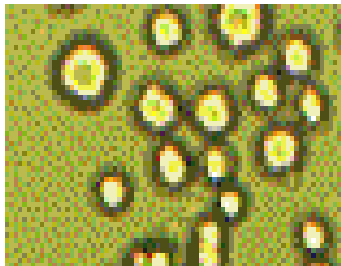
Figure 1. SF9 insect cells showing CPE 24 h infection with WSSV extract from infected shrimp gill tissue.
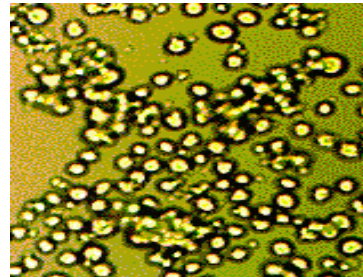
Figure 4: SF9 insect cells showing CPE 96 h post infection with WSSV extract from infected shrimp gill tissue.
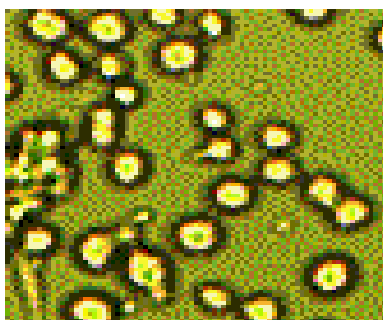
Figure 2. SF9 insect cells showing CPE 48 h post infection with WSSV extract from infected shrimp gill tissue.
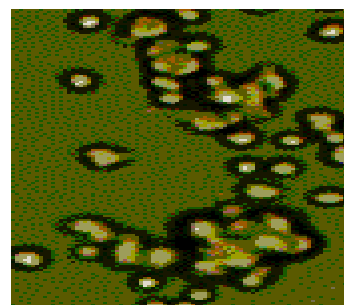
Figure 5. SF9 insect cells showing CPE 120 h post infection with WSSV extract from infected shrimp gill tissue.
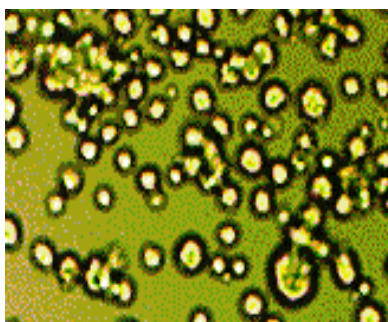
Figure 3. SF9 insect cells showing CPE 72 h post infection with WSSV extract from infected shrimp gill tissue.
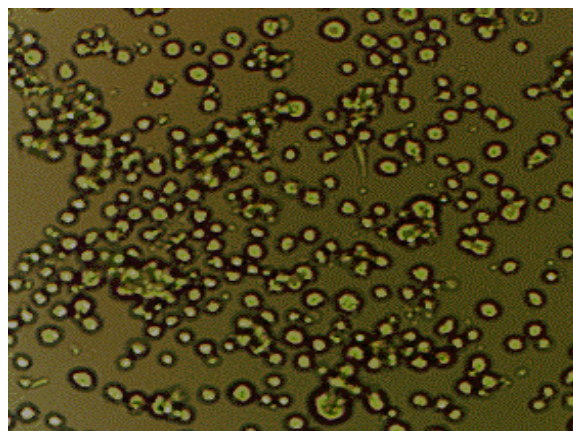
Figure 6. Normal SF9 cells after 4 days.

could be observed in 120 h (Figure 5). Normal SF9 Cells and WSSV Infected SF9 Cells after four days is shown in (Figures 6 and 7).

The infected cell cultures were frozen at -70°C and thawed thrice for harvesting the cells. Cytopathic effect (CPE) was observed in infected cell lines within 2-5 days post infection (PI) in contrast to healthy SF9 cells which served as control. The infected SF 9 cells were swollen and peeled off from the surface of flask, and their cytoplasm was full of particles inside. Nuclei were peripheral, broken and membrane of dead cells was shrunk. Cell mortality has reached approximately 100% after five to six days period. Infectivity assays for WSSV were conducted
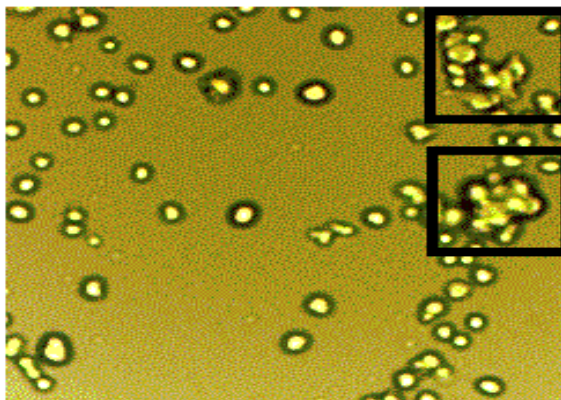
Figure 7. WSSV infected SF9 cells after 4 days.

Table 1. PCR primers for target genes and their amplification size.

Species	Target gene	PCR primer's sequences (5'-3')	Amplicon Size	Reference/genebank accession no
WSSV	WSSV-419 like protein	*WSSV F1:* TGGCATGACAACGGCAGGAGT *WSSV R1:* CGAGCTGCCTTGCCGGAAATTA	550 bp	JN165706 JN165706
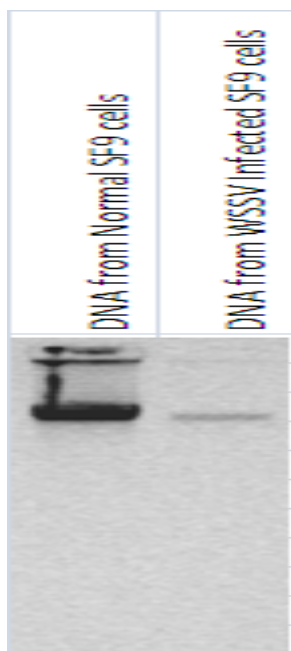
Figure 8. The DNA bands were visualized under ultraviolet transilluminator. Lane 1- DNA from uninfected normal SF9 cells; Lane 2- DNA from WSSV infected SF9 cells.

Infection with WSSV resulted initially in the appearance of focal areas of CPE as early as 2 day post infection (p.i). As the infection progressed, the affected cells rounded up and the CPE extended. Finally, the cells would detached from the surface of the culture vessel resulting in a plaque-like clearing in affected areas. No CPE was observed in the normal SF9 cell culture controls. However, when the cells of SF9 cells was confirmed to be infected by the WSSV cultured for 96-120 h, so many CPE foci (Figures 4 and 5) were observed.

An aliquot of the fluid from the infected culture was used to confirm the presence of WSSV by polymerase chain reaction using PCR primers for target genes (WSSV-419 like protein) and their amplification size (Gene bank accession No: JN165706 & JN165706) is shown in Table 1. The WSSV positive result was shown as 550 bp in SF9 cell line and control sample (Figure 8). The PCR products (6 - 10 µl) were separated by electrophoresis in 2% Agarose gels containing ethidium bromide (1 µg ml^{-1}). The WSSV positive SF9 cell line sample and control showed 550 bp in lanes 2 and 4 (Figure 9). The DNA bands were visualized under ultraviolet transilluminator (Gel Doc 2000, Bio-Rad Laboratories,USA).

DISCUSSION

Research on crustacean viruses is hampered by the lack of continuous cell lines susceptible to them. To overcome this problem, previously challenged immortal insect moth

in vitro using insect SF9 cells cultures prepared from moth insect *Spodoptera frugiperda.*
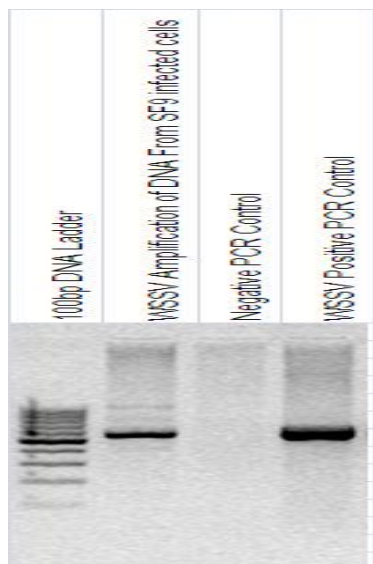
Figure 9. PCR amplification results of WSSV infected cell line; Lane 1- 100 bp DNA ladder (100 - 1000 bp); Lane 2- Insect cell line SF9 sample heavily infected with WSSV; Lane 3- Negative PCR control; Lane 4- WSSV positive PCR control.

and *Spodoptera frugiperda* cell lines with shrimp white spot syndrome virus (WSSV), followed by serial, split-passage of whole cells, showed that this produced cells persistently expressed WSSV antigens (Gangnonngiw et al., 2010). The study was done to determine whether such insect cultures positive for WSSV antigens could be used to infect shrimp *L. vannamei* with WSSV. As such the results are comparable with studies on primary culture of hepatopancreas from *Penaeous monodon* (Uma et al., 2002), *P. orientalis* (Ke et al., 1990), *Palaemontes argentinus* (Sousa et al., 2005), *Marscupenaeus japonicus* (Zilli et al. 2003).

Various studies on initiating primary cell cultures from the heap-topancreas of penaeid shrimp have been reported but with limited success (Toullec et al., 1996; Kasornchandra et al., 1998; Owens and Smith, 1999). Hepatopancreas are more susceptible to most shrimp viruses than other organs and hepatopancreas cell culture will be helpful in isolation viruses (Ke et al., 1990). Mechanical disruption of the tissue was adopted in our study as Toullec (1996) has reported that enzymatic dissociation will not be suitable for fragile hepatopancreas tissue thereby leading to poor attachment and survival of the cells.

The present study showed that susceptibility was observed when monolayer cell sheet derived from normal insect cells SF9 of *L. vannamei* was infected with WSSV. In contrast, other studies reported that *P. stylirostris* and *P. vannamei* Oka cell were susceptible to a non-occluded

baculo-like virus (possibly similar to WSDV) (Lu et al., 1995) and YHV (Tapay et al., 1997) respectively, 2-3 days after incubation. However, this experiment showed that CPE foci were only observed in the cell culture derived from WSSV infected SF9 cells 2-4 days after formation of confluent cell sheet. A similar result was obtained when ovary of *P. monodon* was cultured (Chen et al., 1986). There was survival of the lymphoid tissue and ovary tissue duction of shrimp baculoviruses. Cell lines derived from *Culex tritaeniorhynohurs*, *Culex salinarius*, *Spodoptera frugiperda* and *Armigeres subalbatus* have been exposed to *Baculvoirus penaei* (BP), a related shrimp baculovirus (Summers et al., 1977). Unfortunately, no successful viral infection was obtained in these insect cell lines. As compared to the *in vivo* system, an *in vitro* culture system is equally important for the detailed study of animal viruses. Because no cell lines have been established from shrimp, several insect cell lines have been investigated for the replication and production of shrimp viruses.

The lack of continuous cell lines of shrimps makes us not to be able to fully understand the infections of shrimp pathogenic viruses that often cause serious damage to aquaculture industries. Nevertheless, several investigators have demonstrated that the primary cell cultures were susceptible to certain shrimp viruses (Chen and Kou, 1989; Lu et al., 1995; Tapay et al., 1997). Itami et al. (1999) reported that WSSV induced CPE on primary cultures of lymphoid organs from the kuruma shrimp, *M. japonicus,* within 8 days. In our preliminary study, primary cultures of the SF9 insect cells were also permissive for WSSV infection. Thus, the primary culture system developed in the present study will facilitate large-scale *in vitro* experiments, including bioassay of virus infectivity and biochemical characterization of virus infections. Hence, the recorded observation on the abnormal changes of infected cells in comparison with control cells indicate that the changes are due to CPE produced by WSSV, which was confirmed by PCR of infected cell culture fluid at 550 bp. Similarly, WSSV infected cell culture fluid exhibiting moderate infection are 356 and 232 bp host DNA and WSSV infected cell culture fluid exhibiting heavy infection are 403, 356 and 232 bp host DNA (Uma et al., 2002). From the available reports, this study appears to be the first report on the CPE of WSSV in primary cell culture derived from the insect cells of moth. The inadequacy of the methods for cell line establishment tested in the present study may suggest that more growth factors are needed for the establishment of shrimp virus cell lines.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Optimization of process parameters for α-amylase production under solid-state fermentation by *Bacillus Cereus* MTCC 10202

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An α-amylase producing bacterial strain was isolated from sago factory effluent discharged soil and identified as *Bacillus cereus*. Production of extracellular α-amylase by *B. cereus* was studied in solid-state fermentation. Different substrates like wheat bran, maize bran, corn bran, millet bran, rice bran, greengram bran, blackgram bran, cassava peel powder, cotton seed oil cake, coconut oil cake, sesame oil cake and groundnut oil cake were screened to select the suitable substrate for the production of α-amylase. Among the agro-wastes, wheat bran was found to be the best substrate. Various physical and chemical parameters were optimized. Maximum α-amylase yield was achieved at pH 7 with inoculum level of 10% at 50°C and an incubation period of 72 h. The optimum ratio of substrate to moisture level was found to be 1:2 and substrate weight to flask volume was 1:50. Supplementation of starch at 1% (w/w) concentration and yeast extract at 1.5% (w/w) concentration resulted in maximum production of α-amylase. Calcium chloride at a concentration of 1% (w/w) was found to stimulate α-amylase production. Thus, *B. cereus* produced a high titre of α-amylase in solid state fermentation using inexpensive agro-residue under optimized conditions.

Key words: α-Amylase, *Bacillus cereus*, solid-state fermentation, optimization.

INTRODUCTION

α-Amylases (α-1,4-glucanohydrolases,E.C.3.2.1.1) are endo-amylases that catalyze the hydrolysis of α-D-(1,4) glycosidic linkages in starch components or related carbohydrates, releasing malto-oligosaccharides and glucose in the α-anomeric form (Nazmi et al., 2006). The potential for commercial application of α-amylases is enormous. It is a key enzyme in the conversion of starch to sugar syrups, production of cyclodextrins, preparation of digestive aids, production of chocolates, cakes and fruit juices. They are very extensively used in beverages, baby foods and pharmaceutical industries. Besides their use in starch saccharification, they also find application in brewing, paper and distillery industries (Ramachandran et al., 2004).

α-Amylase has been derived from several fungi, yeasts, bacteria and actinomycetes. However, enzymes from bacterial sources have dominated applications in Industrial sectors. The major advantages of using microorganism for the production of α-amylase are in economical bulk production capacity and they are easy to manipulate to obtain enzymes of desired characteristics. In addition, they allow an economic technology with low resource

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consumption and low emission involving no social or political issues as in the case of animal and plant sources (Venkat, 2007). Microorganisms utilize various substrates as nutrient source for their growth and metabolic activities and subsequently produce metabolism related products. However, fine tuning of nutrient concentrations regulate the microbial metabolism and associated metabolic product formation. Balancing of nutrient concentrations with minimum experimentation and other cultural para-meters is an art in microbial metabolism to optimize enzyme production (Prakasham et al., 2007).

α-Amylase is produced either by submerged (Oyeleke and Oduwole, 2009) or solid-state fermentation (Varalakshmi et al., 2009). Solid-state fermentation domi-nates over submerged fermentation in aspects such as better yield, simple technique, low capital investment, lower levels of catabolite repression, high stability and better product recovery (Babu and Satyanarayana, 1995). Agro-residues are generally considered as the best substrate for the solid-state fermentation processes (Ellaiah et al., 2002). Many agro-industrial by-products such as wheat bran, rice bran, molasses, barley bran, maize meal, soybean meal, potato peel and coconut oil cake have been screened as low cost solid substrates for microbial production of α-amylase in solid-state fermentation (Shukla and Kar, 2006). The major factors that affect microbial synthesis of enzymes in a solid-state fermentation system include the selection of a suitable substrate, microorganism, inoculum concentration, parti-cle size and moisture level of the substrate. The aim of the present study is to investigate the production of α-amylase from *Bacillus cereus* under solid-state fermen-tation using inexpensive and abundantly available agro-residues as substrate thereby reducing the cost of enzyme production.

In this paper we have reported the factors that influence maximization of α-amylase production by *B. cereus.*

MATERIALS AND METHODS

Screening and isolation of α-amylase producing bacteria

A total of 433 bacterial isolates were isolated from different sources including field soils, waste water discharged soils, effluents and spoiled food sources by serial dilution technique. The bacterial isolates were screened for α-amylase production on starch agar plates. The hydrolysis zone formed on starch agar plates were visualized by flooding the plates with Gram's iodine solution. The amylolytic potential was estimated using the amylolytic Ratio (R/r) defined as the diameter of the hydrolyzation zone (R) divided by the diameter of the producing colony (r) (Bernhardsdotter et al., 2005). Isolates having a higher amylolytic ratio (above 3) of clearing zone to colony size were grown in liquid broth and the amount of amylase production was determined from cell free supernatant. The bacterial isolate that produced maximum amylase was selected and identified in the Institute of Microbial Technology (IMTECH) at Chandigarh, India. The selected bacterial strain was maintained in nutrient agar slants. The bacterial strain was sub-cultured periodically after every 30 days and stored at 4°C.

Preparation of bacterial inoculum

A volume of 50 ml nutrient broth supplemented with 1% soluble starch was taken in a 250 ml Erlenmeyer flask. The flask was sterilized in an autoclave at 15 lb pressure (121ºC) for 15 min. After cooling the medium, a loopfull of bacteria from a 24 h old slant was aseptically transferred to the flask and kept at 37°C in a rotary shaker (150 rpm). After 24 h of incubation, 1 ml of this nutrient broth culture was used as the inoculum for 100 ml of production medium.

Production of bacterial α-amylase in solid-state fermentation

Solid-state fermentation (SSF) was carried out in 250 ml Erlenmeyer flasks containing 5 g of wheat bran. The substrate was moistened with 10 ml of distilled water, autoclaved (15 lb) at 121°C for 15 min and cooled. The flasks were inoculated with 1% (v/w) bacterial inoculum and incubated at 37°C for 72 h.

Enzyme extraction

The enzyme from the fermented bacterial bran was extracted twice with 50 ml of 10 mM phosphate buffer (pH 7.0). Extraction was done by soaking the fermented solids with phosphate buffer for 30 min at 30°C on a rotary shaker (150 rpm). The slurry was squeezed through a damp cheese cloth. The extracts were pooled and centrifuged at 4°C for 15 min at 5000 rpm to separate small wheat bran particles, cells and spores. The brown clear supernatant was used as the source of α-amylase (Ellaiah et al., 2002).

α-Amylase activity

α-Amylase activity was determined as per the method described by Bernfeld (1955). One unit of α-amylase activity is defined as the number of μmol of maltose liberated by 1 ml of enzyme solution per minute. Amylase production was expressed as units per gram of dry substrate.

Optimization of process parameters for α- amylase production

Various process parameters such as inoculum level, incubation period, moistening agents, moisture level, pH, temperature, carbon sources, nitrogen sources and metal salts were optimized. The strategy followed was to optimize each parameter, independent of the others and subsequently optimal conditions were employed in all experiments.

Effect of different agro-residues as solid substrates

The bacterial strain was inoculated (1%v/w) in 250 ml Erlenmeyer flasks each containing 5 g of various substrates (wheat bran, maize bran, corn bran, millet bran, rice bran, greengram bran, blackgram bran, cassava peel powder, cotton seed oil cake, coconut oil cake, sesame oil cake and groundnut oil cake. The flasks were moistened with 10 ml of distilled water and incubated at 37°C for 72 h. The enzyme was then extracted and assayed for α-amylase activity. The best solid substrate achieved by this step was used for subsequent experiments.

Effect of inoculum level

Culture flasks (250 ml) each containing wheat bran (5 g) moistened with 10 ml distilled water were autoclaved and inoculated with dif-

ferent amounts (2, 4, 6, 8, 10 and 12% v/w) of bacterial inoculum. All the flasks were incubated at 37°C for 72 h. The contents of the flasks were harvested and assayed for α-amylase activity. The optimum inoculum level achieved was used for further optimization studies.

Effect of incubation period

The inoculated flasks were incubated at different time intervals such as 24, 48, 72, 96 and 120 h. After each incubation period, the enzyme was extracted and assayed for α-amylase activity. The optimum incubation period found was followed for further experiments.

Effect of moistening agents

The effect of moistening agents (MA) on the production of α-amylase was studied using the following five mineral salt solutions prepared in distilled water as follows:

MA I (g/L) - $MgSO_4$ $7H_2O$ - 0.5; K_2HPO_4 -1.5 - pH 7.2
MA II (g/L) - KH_2PO_4 - 11.0; NaH_2PO_4 - 6.1; KCl - 3.0; $MgSO_4$ $7H_2O$ - 0.1-pH 7.2
MA III (g/L) - K_2HPO_4 - 0.1; $(NH_4)H_2PO_4$ -1.0; $MgSO_4$ $7H_2O$ - 0.5; $CaCl_2$ -0.1; $FeSO_4$,- 0.1; $MnSO_4$ - 0.1- pH 7.2
MA IV (g/L) - $(NH_4)NO_3$ - 2.0; K_2HPO_4 - 5.0; $MgSO_4$ $7H_2O$ - 0.5; KCl - 0.5 - pH 7.2
MA V (g/L) - KH_2PO_4 - 11.0; NaH_2PO_4 - 6.1; KCl- 3.0; $MgSO_4$ $7H_2O$ - 1.0 - pH 7.2

Besides the aforementioned moistening agents, distilled water and tap water were also used as moistening agents. The enzyme was extracted and assayed from each set. The selected moistening agent was used to moisten the substrates in the subsequent experiments.

Effect of moisture level

The effect of different moisture level on α-amylase production was studied by varying the ratio of weight of the substrate to the volume of moistening agents (1:1.0, 1:1.5, 1:2.0, 1:2.5 and 1:3.0 w/v). The fermentation was carried out at 37°C for 72 h having other experimental conditions at their optimum levels. The optimum ratio of weight of solid substrate to volume of moistening agent achieved by this step was fixed for subsequent studies.

Effect of initial pH

To determine the effect of initial pH on α-amylase production, the pH of the moistening agent was varied from 4 to 10 with one unit interval using 0.1 N HCl and 0.1 N NaOH. An inoculum level of 10% and moisture ratio of 1:2 was employed. The fermentation was carried out at 37°C for 72 h. After the incubation period, the enzyme was extracted and assayed. The optimum initial pH of the solid substrate determined by this step was fixed for subsequent experiments.

Effect of incubation temperature

The fermentation was carried out at various temperatures such as 30 to 90°C with an interval of 10°C, keeping all other parameters at their optimum levels. After incubation, the enzyme was extracted and assayed for α-amylase activity.

Effect of selected supplementary carbon sources

The effect of various carbon supplements on α-amylase production was evaluated by adding carbon sources (1% w/w) such as starch, maltose, lactose, sucrose, glucose and fructose. The crude enzyme extract was analyzed for α-amylase activity. The most suitable carbon supplement was found to be starch.

Effect of different concentrations of starch

Wheat bran was supplemented with starch at different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5% w/w) individually. All the other experimental conditions were kept at their optimum. The optimum starch concentration determined was used for further studies.

Effect of selected supplementary nitrogen sources

Various nitrogen sources such as yeast extract, peptone, tryptone, casein, beef extract, urea, ammonium sulphate, ammonium chloride and sodium nitrate at 1% (w/w) concentration was incorporated separately into the fermentation medium to select the best nitrogen supplements for α-amylase production. α-Amylase activity was determined in the cell free supernatant. The most suitable nitrogen supplement was found to be yeast extract.

Effect of different concentrations of yeast extract

Different yeast extract concentrations such as 0.5, 1.0, 1.5, 2.0 and 2.5% (w/w) were supplemented in the fermentation medium separately. All the other experimental conditions were kept at their optimum.

Effect of the amount of substrate to flask volume

The effect of the amount of substrate to flask volume was studied by changing the ratio of weight of the substrate to flask volume. Different amounts (5, 10, 15, 20 and 25 g) of wheat bran were taken individually in 250 ml conical flasks. The experiment was conducted at 50°C for 72 h keeping all other conditions at their optimum levels. The optimum ratio of the amount of substrate to flask volume achieved by this step was fixed for subsequent experiments.

Effect of different metal salts on α-amylase production

Influence of different metal salts on α-amylase production was studied by incubating the culture medium with various metal salts, namely, calcium chloride, magnesium sulphate, ferric chloride, manganese sulphate, copper sulphate, mercuric chloride, zinc sulphate, silver chloride, sodium chloride, potassium chloride, lead nitrate and lithium sulphate each at a concentration of 1 mM was mixed with the moistening agent individually (Sharma et al., 2007). After incubation, α-amylase was extracted and assayed for its activity.

Effect of different concentrations of calcium chloride

Wheat bran was incorporated with various concentrations of calcium chloride separately (0.2, 0.4, 0.6, 0.8 and 1.0% w/w) and fermentation was carried out under optimized conditions. After incubation period, the enzyme was extracted and assayed.

Table 1. α-Amylase production on selected agro-residues as substrates.

Agro-residue	α-Amylase activity (U/gds)
Wheat bran	24.85±0.98
Maize bran	17.42±1.20
Corn bran	9.35±1.33
Millet bran	14.56±1.17
Rice bran	18.55±0.58
Greengram bran	12.92±1.00
Blackgram bran	16.57±1.02
Cassava peel powder	20.62±0.90
Cotton seed oil cake	13.87±0.80
Coconut oil cake	20.03±0.52
Sesame oil cake	7.47±1.01
Groundnut oil cake	15.95±0.73

U/gds, Units per gram of dry substrate

RESULTS AND DISCUSSION

Isolation and identification of the selected amylolytic bacterial strain

Among the 433 bacterial isolates, 42 showed amylolytic ratio greater than 3 (good), 209 showed between 2 to 3 (moderate) and 182 showed less than 2 (poor). Bernhardsdotter et al. (2005) and Oyeleke and Oduwole (2009) have recorded an amylolytic ratio of 3 for *Bacillus* sp. isolate L 1711 and 3.1 for *Bacillus subtilis* respectively. The bacterial isolates were grown in liquid broth and the amount of α-amylase production was determined in cell free supernatant. Bacterial strain isolated from sago factory effluent discharged soil showed maximum α-amylase activity (36.85 U/ml) and was selected as the best potent producer of α-amylase. The selected bacterial isolate was identified as Gram positive, rod shaped and non-motile with the size ranging from 2 to 3 μm. Off-white colonies were produced on nutrient agar medium. The biochemical characterization of the isolate showed positive results for methyl red, voges proskauer, nitrate reduction, catalase, oxidase, gelatin and starch hydrolysis; negative results for indole test, citrate utilization and urea hydrolysis. Regarding carbohydrate fermentation test, the selected bacterial isolate produced acid with glucose and maltose, whereas there was no acid production with lactose, sucrose, mannitol and xylose as substrates. Based on these morphological, physiological and biochemical tests, the selected bacterial strain was identified as *B. cereus* at the Institute of Microbial Technology (IMTECH), Chandigarh, India and deposited in their culture collection with the accession number MTCC 10202.

Many workers have reported *Bacillus* sp. as a potent producer of α-amylase (Sodhi et al., 2005; Anto et al.., 2006; Thippeswamy et al., 2006; Asgher et al., 2007;

Ahmed, 2008).

Optimization of various physical and chemical parameters for α- amylase production

Effect of different agro-residues as solid substrate

In SSF process, the selection of a suitable solid substrate for fermentation process is a critical factor and thus involves the screening of a number of agro-wastes for microbial growth and product formation. Various agro-residues as substrates were screened for amylase production and the results are presented in Table 1. All the substrates supported growth and enzyme production. A high titre of α-amylase activity was obtained with wheat bran followed by cassava peel powder and coconut oil cake. The results of the present study are in concurrence with Baysal et al. (2003), Anto et al. (2006) and Gangadharan et al. (2006), who have reported wheat bran as the best substrate for optimal production of amylase by *B. subtilis*, *B. cereus* MTCC 1305 and *Bacillus amyloliquefaciens*, respectively. Wheat bran was found to be the suitable substrate for α-amylase production when compared to all the other agro-residues. This might be due to the presence of sufficient nutrients and ability to remain loose even under moist conditions, thereby providing a large surface area (Babu and Satyanarayana, 1995). In the subsequent experiments, wheat bran was used as the substrate for the production of α-amylase.

Effect of inoculum level

The fermentation profile of an organism is usually affected by the initial inoculum concentration. An increase in inoculum level was found to improve the growth and production of α-amylase and reached maximum at 10% v/w (29.74 U/gds). Increase in inoculum level above 10% was found to decline the enzyme production (Figure 1a). This might be due to higher concentration of inoculum resulting in increased competition for carbon source and nutrients, which might lead to exhaustion of nutrients. This imbalance would have resulted in reduced enzyme production and also the free excess liquid present in an unabsorbed form would have given rise to an additional diffusional barrier together with that imposed by the solid nature of the substrate might lead to decrease growth and enzyme production (Baysal et al., 2003). Lower inoculum level would have resulted in a lesser number of cells in the production medium. These require a longer time to grow to optimum number to utilize the substrate and form the desired product (Kashyap et al., 2002). The results of the present study is in concurrence with Anto et al. (2006) who have reported 10% (v/w) as optimum inoculum concentration for the production of amylase with *B. cereus* MTCC 1305.
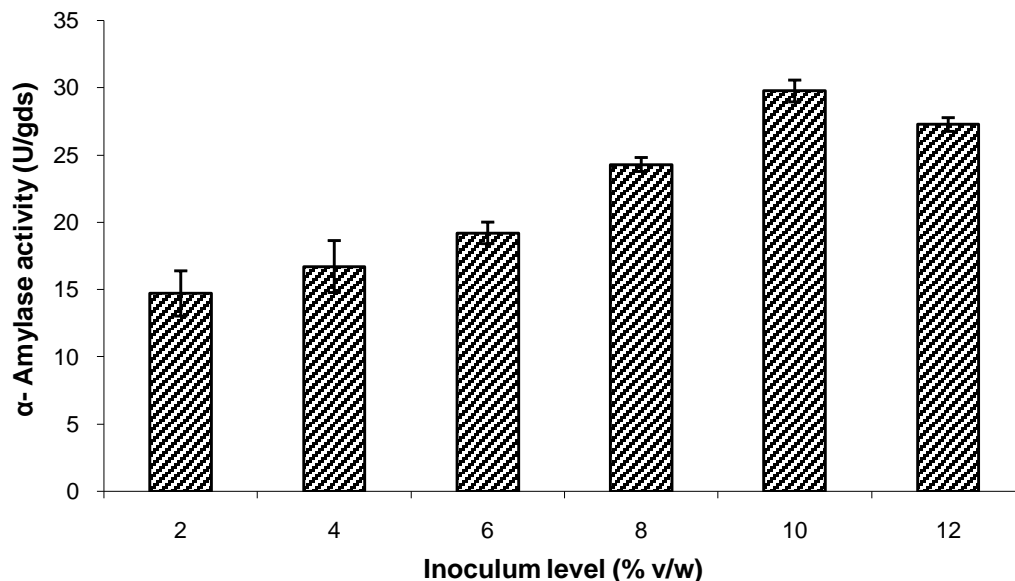
Figure 1a. Effect of inoculum level on α- amylase production.
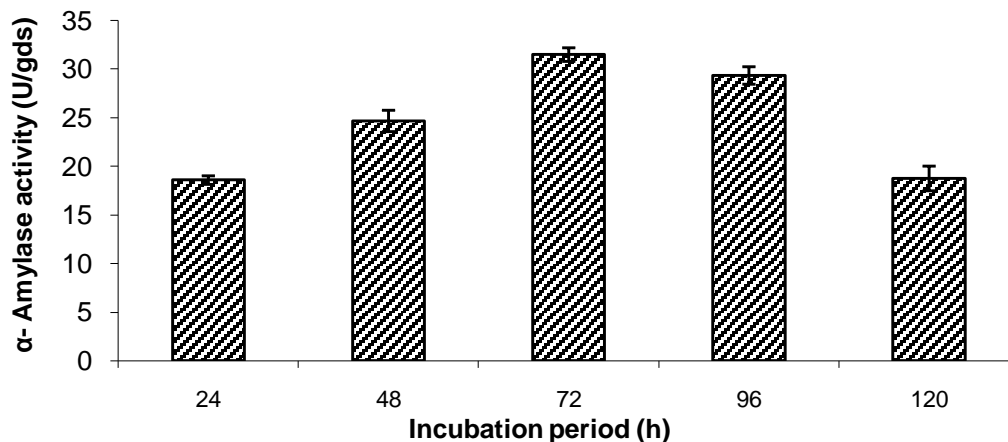
Figure 1b. Effect of incubation period on α- amylase production.

In contrast, Baysal et al. (2003) have reported that 20% inoculum led to good enzyme production with *B. subtilis*. An inoculum concentration of 10% (v/w) was fixed for further optimization experiments.

Effect of incubation period

The optimum incubation time for achieving the maximum enzyme production is governed by the characteristics of the culture and is based on the growth rate of the microorganisms and its enzyme production pattern. α-amylase production reached the peak (31.50 U/gds) at 72 h of incubation (Figure 1b). The enzyme production showed a gradual decrease on further extension of fermentation period. This could be due to loss of moisture or denaturation or decomposition of the enzyme resulting in variation in pH during fermentation or due to interaction with other components in the culture medium (Ahmed, 2008). The observed results are in agreement with Babu and Satyanarayana (1995) and Anto et al. (2006) who have reported maximum amylase production at 72 h of incubation with *Bacillus coagulans* and *B. cereus* MTCC 1305, respectively; whereas, Baysal et al. (2003) have reported 48 h as optimum incubation time for *B. subtilis*. In most cases, the optimum incubation period for α-amylase production in SSF using *Bacillus* sp. varied from 48 to 72 h, depending on the environmental conditions (Sivaramakrishnan et al., 2006). An incubation period of 72 h was followed for further experiments.

Table 2. Effect of moistening agents on α-amylase production.

Moistening agent	α- Amylase activity (U/gds)
MA I	34.93±0.39
MA II	30.76±0.15
MA III	15.51±0.95
MA IV	25.67±0.62
MA V	14.94±1.05
Tap water	21.40±0.55
Distilled water	22.01±0.43

U/gds, Units per gram of dry substrate. Values are mean of three replicates ± SD.

Table 3. Effect of moisture level on α-amylase production.

Moisture level (substrate: MA I) (w/v)	α- Amylase activity (U/gds)
1:1.0	24.28±0.52
1:1.5	29.35±0.89
1:2.0	36.66±0.11
1:2.5	28.99±1.66
1:3.0	25.88±0.43

U/gds, Units per gram of dry substrate. Values are mean of three replicates ± SD.

Effect of moistening agents

The nature of the moistening agent (MA) is a main factor in SSF which often determine the success of a process. MA I resulted in maximum (Table 2) α-amylase production. Similar observations were made by Babu and Satyanarayana (1995) who have reported mineral salt solution as the best moistening agent for *B. coagulans* in SSF. In contrast, Sodhi et al. (2005) have reported that amylase production by *Bacillus* sp. PS - 7 was maximum when tap water was used as moistening agent. MA I was used to moisten the substrate wheat bran, in the subsequent optimization experiments.

Effect of moisture level

The importance of moisture level in SSF and its influence on the biosynthesis of enzymes has been attributed to the interference of moisture in the physical properties of solid particles. The enzyme production attained a peak, when moistened with MA I in the ratio of 1:2 (Table 3). Enzyme production was found to decrease with lower moisture content. This might be due to an insufficient water availability preventing a good diffusion of solutes and gas, thereby resulting in slow down or complete arrest of cell metabolism. Also, it leads to lower degree of substrate swelling and higher water tension and thereby

reduces the solubility of nutrients (Gervais and Molin, 2003). There was a decline in α-amylase production when the moisture level was increased above 1:2. It was suggested that higher moisture levels decreased porosity, promoted development of stickiness, reduced gas volume and exchange, changed substrate particle structure, resulted in static hindrance of the growth of the organisms through reduction in inter particle spaces, decreased diffusion and impaired oxygen transfer (Perez-Guarre et al., 2003). It also resulted in poor adsorption of enzyme to the substrate particle (Swain and Ray, 2007). The observed results are in agreement with the results of Babu and Satyanarayana (1995) and Anto et al. (2006) who have reported an optimum ratio of 1:2 for *B. coagulans* and *B. cereus* MTCC 1305, respectively in SSF; whereas, Sodhi et al. (2005) have reported maximum α-amylase production when the ratio of wheat bran to moisture level was maintained at 1:1.5 for *Bacillus* sp. PS - 7.

The moisture content of the medium changes during fermentation which is a result of evaporation and metabolic activities and so adjusting the optimum moisture level of substrate during SSF is considered most important. Hence, the substrate: initial moisture level ratio was fixed as 1:2 for the subsequent experiments.

Effect of pH

High enzyme titre (34.93 U/gds) was attained when the initial pH of the medium was maintained at 7 (Figure 2a). pH is one of the important factors that determine the growth and enzyme secretion of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. As the metabolic activities of the microorganisms are very sensitive to changes in pH, α-amylase production was found to be affected when the pH level is above or below the optimum pH. The obtained optimum pH value is in concurrence with earlier reports of Konsula and Kyriakides (2004) and Asgher et al. (2007) with *B. subtilis.* Thippeswamy et al. (2006) and Mishra and Behera (2008) have reported maximum amylase production near neutrality for *Bacillus* sp. However, Aiyer and Modi (2005) reported pH 9 to be optimum for the production of amylase by *Bacillus licheniformis* SPT 27.

Effect of incubation temperature

Temperature control in the substrate bed is very important for SSF since growth and production of enzymes or metabolites are usually sensitive to temperature. Optimal temperature for maximum α-amylase production was found to be at 50°C (31.77 U/gds). A reduction in enzyme activity was observed at temperatures above 50°C (Figure 2b). Temperatures above 50°C resulted in lesser growth of *B. cereus* and decrease
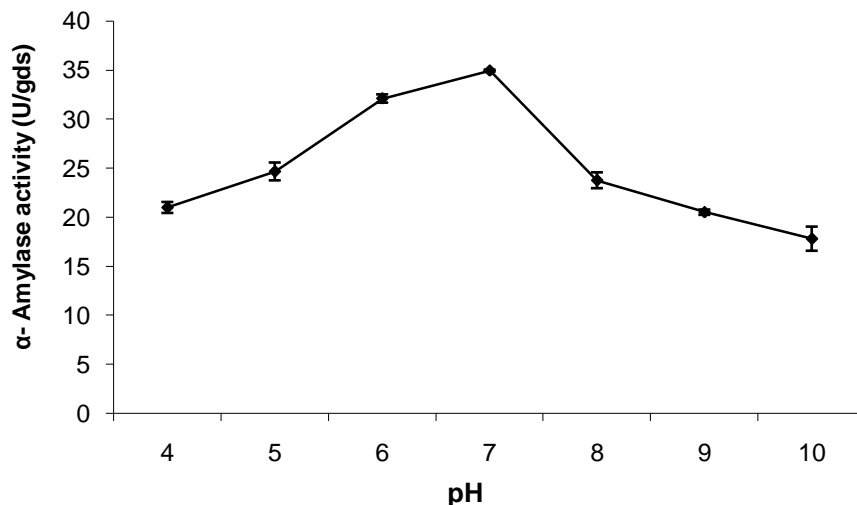
Figure 2a. Effect of initial pH on α-amylase production.
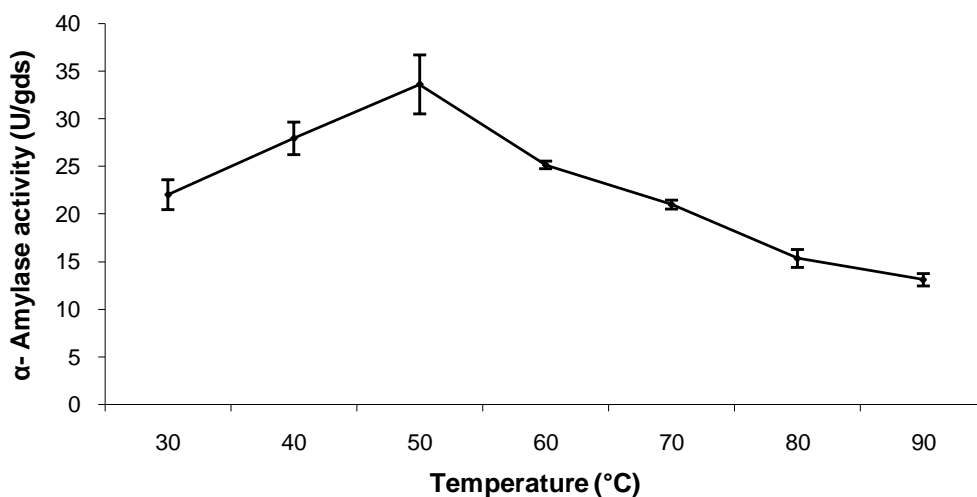
Figure 2b. Effect of temperature on α-amylase production.

in the yield of enzyme. Moisture loss in solid substrate due to increase in temperature might be the cause for lesser production of α-amylase. An identical temperature optimum for α-amylase production was reported by Asgher et al. (2007) who have reported 50°C to be the optimum temperature for amylase production by *B. subtilis* JS- 2004 strain. However, Konsula and Kyriakides (2004), Goyal et al. (2005) and Sodhi et al. (2005) have reported maximum amylase production at 40°C by *B. subtilis*, *Bacillus* sp. I-3 and *Bacillus* sp. PS - 7, respectively.

Effect of selected supplementary carbon sources

Growth and enzyme production of any organisms are greatly influenced by the nutrients available in the growth medium. α-amylase is an inducible enzyme. The carbon sources in the medium are found to exert a profound effect on the enzyme production behaviour. Some carbon sources supported good growth with low enzyme production while others supported good growth as well as enzyme secretion. Among the different carbon supplements examined, starch promoted high enzyme titre (48.53 U/gds) which was followed by maltose, lactose and sucrose (Figure 3a). The results are in agreement with the reports of Konsula and Kyriakides (2004) for *B. subtilis*, Goyal et al. (2005) for *Bacillus* sp. I-3 and Sodhi et al. (2005) for *Bacillus* sp. PS-7 who have reported maximum amylase production when starch was used as carbon supplement. Glucose and fructose supplementation resulted in the repression of enzyme
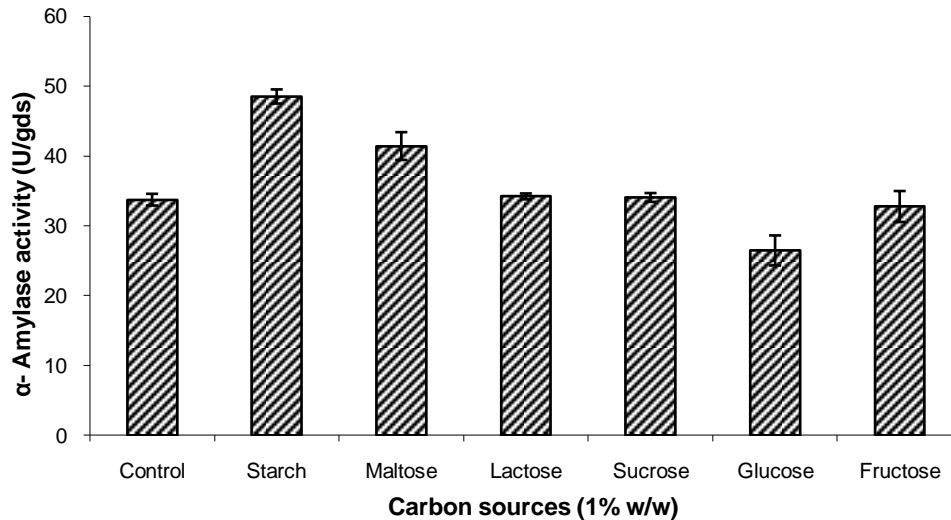
Figure 3a. α-Amylase production using selected supplementary carbon sources.
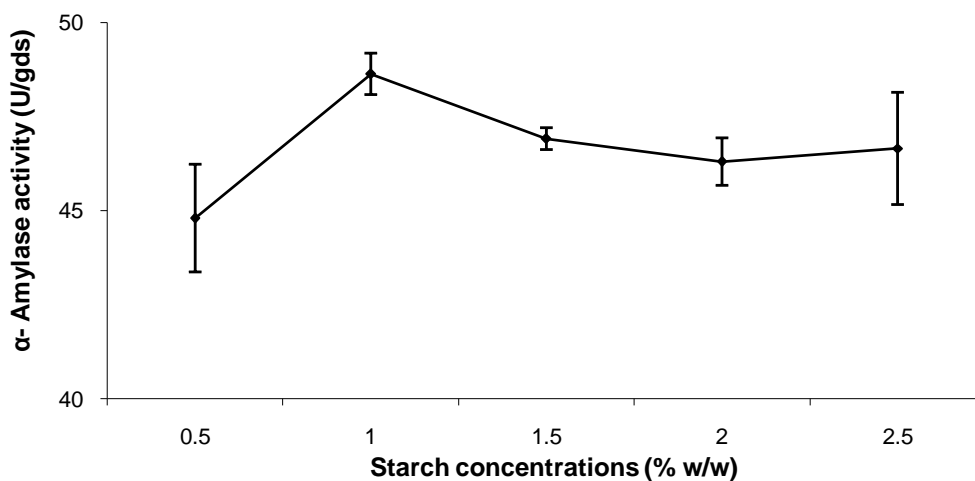
Figure 3b. α-Amylase production at different starch concentrations.

production. This might be due to the feedback inhibition caused by the presence of glucose and fructose as reported by Rama and Srivastav (1995). The repression is higher with glucose than fructose. Glucose acted as a catabolic repressor for the enzyme production.

Effect of different concentrations of starch

Among the different concentrations of starch, 1% resulted in maximum increase in α-amylase production (48.63 U/gds). The observed results (Figure 3b) are similar to the report of Gangadharan et al. (2006) for *B. amyloliquefaciens*. In contrary, Mishra and Behera (2008) observed that addition of 2% starch resulted in high enzyme yield by *Bacillus* sp.

Effect of selected supplementary nitrogen sources

Added nitrogen sources have been reported to have an inducing effect on the production of various enzymes in SSF system. Among the various organic and inorganic nitrogen sources tested (Figure 4a), yeast extract showed maximum α-amylase yield (55.06 U/gds) followed by peptone, beef extract, tryptone, casein and urea. The influence of yeast extract on α-amylase production might be due to the presence of vitamin B group (promoting group), free amino acids and carbohydrate. It is observed that organic nitrogen compounds increased α-amylase production than when compared to inorganic compounds. Same observation was made by Qader et al. (2006) for *Bacillus* sp. AS-1. The obtained result is in concurrence with the work reported earlier by Thippeswamy et al.
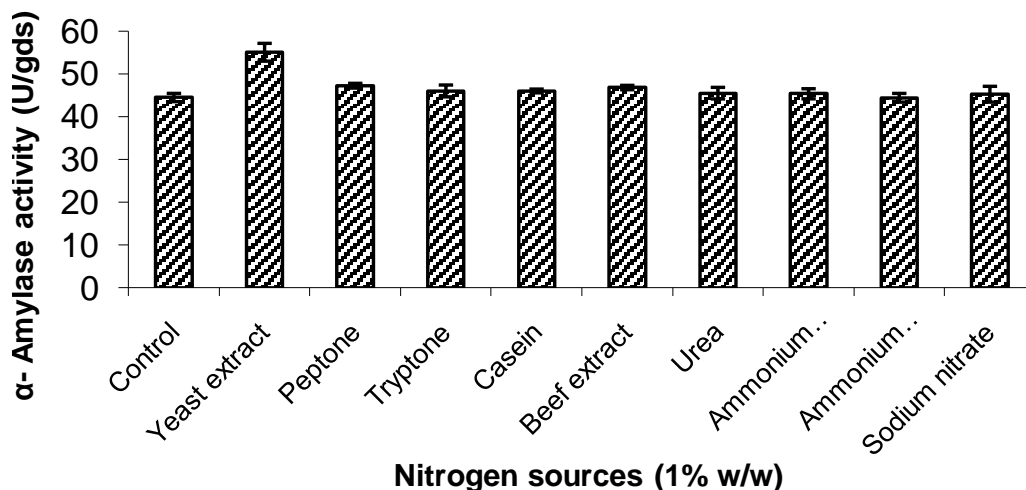
Figure 4a. α-Amylase production by selected supplementary nitrogen sources.
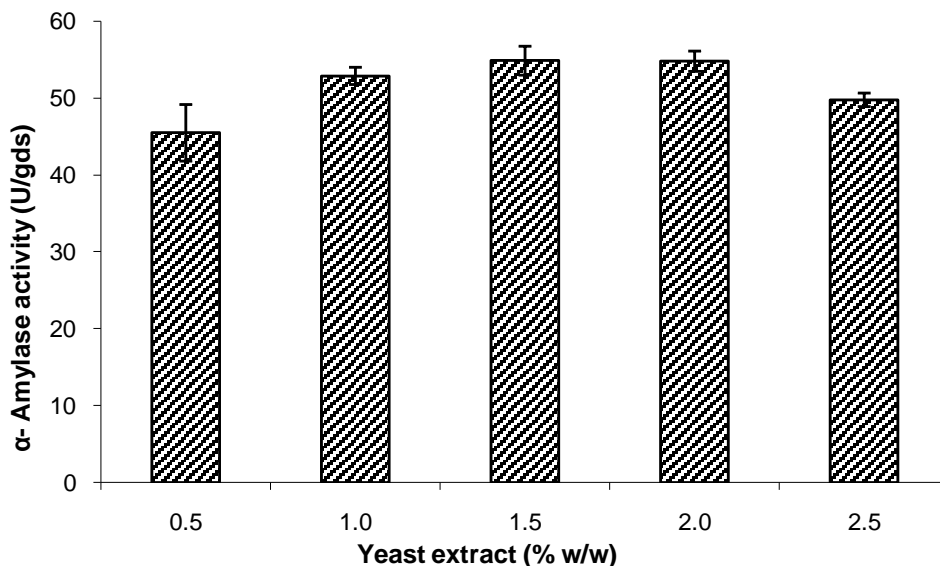
Figure 4b. α-Amylase production at different yeast extract concentrations.

(2006) for *Bacillus* sp. and Asgher et al. (2007) for *B. subtilis* JS 2004 who have reported yeast extract as the best nitrogen supplement for amylase production.

Effect of different concentrations of yeast extract

The α-amylase yield reached the peak at 2.0% concentration of yeast extract. Above 2.0%, there was a decrease in α-amylase yield (Figure 4b), which might be due to changes in C/N ratio. The nitrogen is metabolized to produce primarily amino acids, nucleic acids, protein and cellular components. Low levels of nitrogen are inadequate for enzyme production; whereas, excess

nitrogen can equally be detrimental causing in some cases a complete inhibition of enzyme production (Kole et al., 1988). The observation recorded in this study is similar to the earlier report of Hamilton et al. (1999) who have recorded maximum amylase yield at 2.0% yeast extract concentration for *Bacillus* sp. IMD 435.

Effect of amount of substrate to flask volume

α-Amylase production was found to be maximum when the ratio of substrate weight -to- flask volume was 1:50 (Figure 5a). This finding is in agreement with the reports of Satyanarayana (1994) for amylase production by *B.*
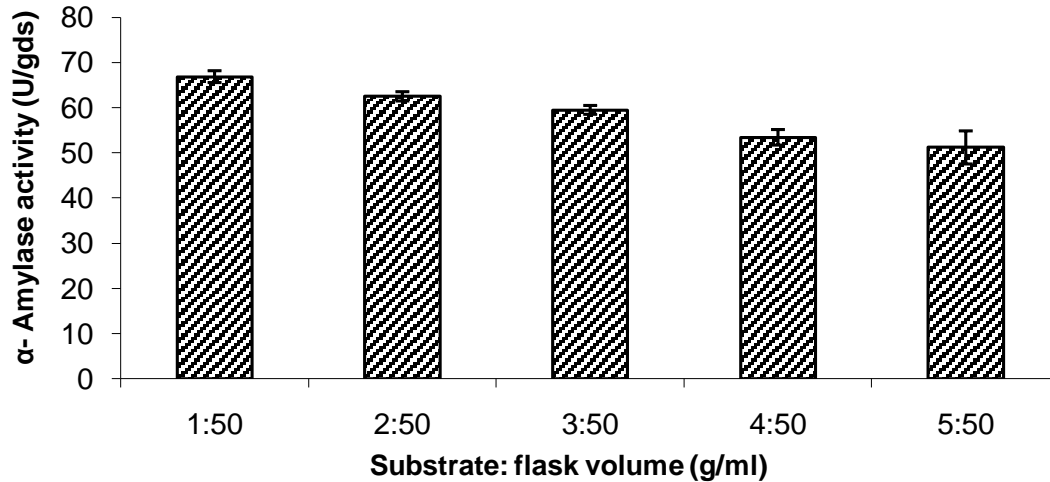
Figure 5a. Effect of amount of substrate to flask volume.

Table 4. Effect of metal salts on α-amylase production.

Metal salt (1 mM in moistening agent)	α- Amylase activity (U/gds)
Control	48.69±3.17
Calcium chloride	65.89±1.99
Magnesium sulphate	61.69±1.70
Ferric chloride	55.59±0.84
Manganese sulphate	56.01±0.95
Copper sulphate	44.33±4.19
Mercuric chloride	41.52±3.21
Zinc sulphate	48.46±2.12
Silver chloride	53.34±2.07
Sodium chloride	56.71±1.81
Potassium chloride	54.44±1.64
Lead nitrate	44.92±5.29
Lithium sulphate	61.28±2.21

Control, without metal salts. U/gds, units per gram of dry substrate. Values are mean of three replicates ± SD.

coagulans.

Effect of metal salts on α-amylase production

Supplementation of salts of certain metal ions is found to influence the growth of microorganisms and thereby stimulate or inhibit enzyme production. Among the metal ions supplemented (Table 4), $CaCl_2$ increased α-amylase production. $MgSO_4$, $LiSO_4$, NaCl, $MnSO_4$, $FeCl_2$, $AgCl_2$ and KCl stimulated α-amylase production; whereas, $PbNO_3$, $HgCl_2$, $CuSO_4$ and $ZnSO_4$ had negative effect on amylase production. α-Amylases are known to be metalloenzymes, Ca^{2+} ions are reported to be present in majority of these enzymes. Addition of $CaCl_2$ to the fermentation media increased the enzyme production.

This might be due to the increased availability of calcium ions (Francis et al., 2003). The results of present study are in line with Asgher et al. (2002) who have recorded higher amylase production on addition of calcium chloride in the medium for *B. subtilis.* However, Goyal et al. (2005) have reported that $LiSO_4$ and $CaCl_2$ in the medium increased amylase production in *Bacillus* sp. I-3.

Effect of different concentrations of calcium chloride

The production of α-amylase is found to be calcium dependent. The α-amylase yield increased with the addition of calcium chloride and peaked (64.97 U/gds) with 1% (Figure 5b). Asgher et al. (2002) have recorded maximum amylase yield with 0.05% calcium chloride supplemen-
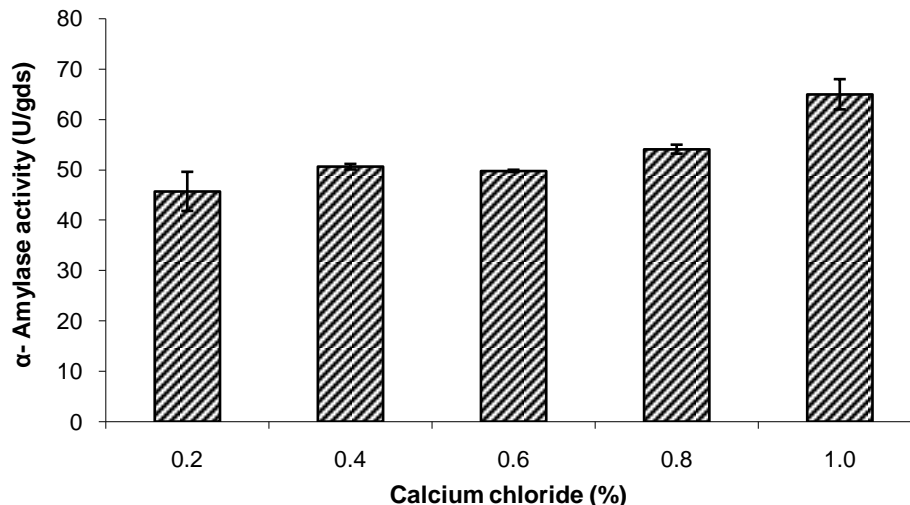
Figure 5b. Effect of different concentrations of calcium chloride.

tation by *B. subtilise*; whereas, Qader et al. (2006) have reported that a low level of calcium chloride (0.02%) was sufficient for amylase production by *Bacillus* sp. AS-1. The effect of metal ions on α-amylase production varies from microorganism to microorganism. Hence, based on the present observations, calcium chloride at a concentration of 1% was supplemented for maximum α-amylase production.

Conclusions

The present study describes the suitability of the laboratory isolate *B. cereus* for the commercial exploit-tation using simple, less expensive and economically feasible substrate, wheat bran with the supplementation of simple nutrients like starch and yeast extract. The maximum productivity of α-amylase was achieved by optimized process parameters such as 10% inoculum concentration, incubation time of 72 h, incubation tem-perature at 50°C, 1:2 (w/v) ratio of weight of wheat bran to salt solution, 1:50 ratio of substrate weight to flask volume and pH 7. The scope of the study may be explored in large scale for industrial application.

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Full Length Research Paper

Microbial diversity and performance of an innovative micro-aerobic bioreactor

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The treatment performance of two parallel continuous single stage activated sludge bioreactor: an aerobic bioreactor (ABR) operated at dissolved oxygen (DO) levels of 3-4 mg/L, and an innovative micro-aerobic bioreactor (MABR) operated at DO levels of 0.4-0.8 mg/L and the microbial diversity of MABR was investigated and compared. Under the similar conditions, at a hydraulic time (HRT) of 25 h, an organic loading rate (OLR) of 3.4 ± 0.1 kg COD /m^3·d, the influent total phosphate (TP) was 44 ± 2 mg/L. The COD removal efficiencies of the MABR and the ABR were 93 ± 3 and $90\pm3\%$, respectively, and no significant difference in performance was noted between the two systems (P=0.087>0.05). It was, however, noted that TP removal efficiency of the MABR ($62\pm4\%$) was significantly higher than that of ABR ($43\pm2\%$) (P=0.00). Phylogenetic analysis indicated that bacteria in the MABR was highly diverse. It is likely that facultative anaerobes, microaerophile and aerobes were able to coexist in the MABR. These findings might be helpful in developing an economic treatment system, which can be a better alternative for treatment of many pollutants requiring aerobic/anaerobic sequential treatment.

Key words: Biodiversity, micro-aerobic bioreactor, phosphorous removal, phylogenetic analysis.

INTRODUCTION

Many xenobiotic compounds which are refractory under aerobic conditions seem to be readily biotransformed anaerobically (Zitomer and Speece, 1993). In turn, partially degraded products which resist further anaerobic degradation can be completely mineralized by aerobic microorganisms (Long et al., 1993). These facts point out the limitation and complementary of aerobic and anaerobic treatments and lead to the development and application of anaerobic-aerobic sequence processes. In an anaerobic-aerobic sequence system, the anaerobic and aerobic bacteria function in separate units that complement each other.

An economical strategy of operating anaerobic-aerobic systems would be to construct micro-ecosystems integrating anaerobic and aerobic niches and creating synergism between reductive and oxidation catabolisms. The coexistence of anaerobes and aerobes in aerobic and anaerobic environments indicates the possibility of constructing microecosystems in which both anaerobes and aerobes are able to survive (Lens et al., 1995). Under O_2-limited conditions, aerobic respiring microorganisms can maintain very low dissolved oxygen concentrations in the aerobic/anaerobic cocultured system, and the eventual inhibition of anaerobes is avoided. Considerable research interest is currently being focused on investigations into micro-aerobic conditions in natural environ-

1 feed tank
2 metering pump
3 influent
4 valves
5 porous stone diffuser
6-8 sampling ports
9 effluent
10 air pump
11 air flow meter
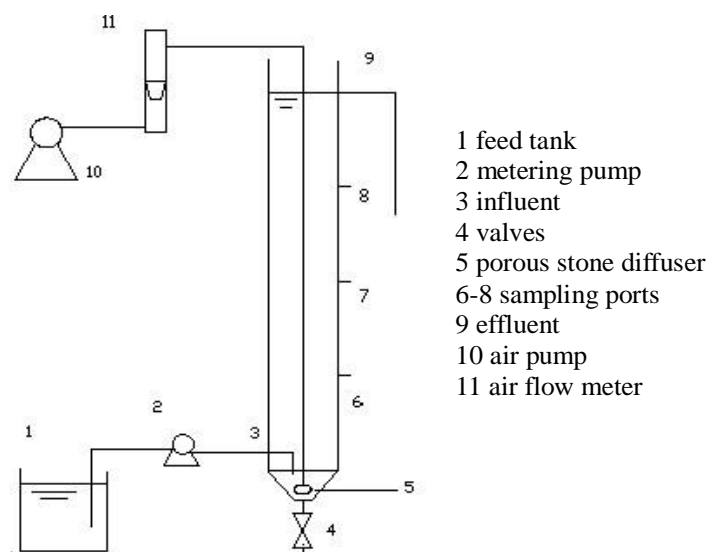
Figure 1. The scheme of the reactor.

ments (Pitcher et al., 2002), in the fields of wastewater treatment (Lasik and Nowak, 2007), biotech-nological fermentation (Li et al., 2010), bioremediation of toxic compounds (Franciscon et al., 2010) and cultivation of new bacterial species (Geelhoed et al., 2009). Micro-aerobic conditions may be defined as the transition condition, making use of aerobic and anaerobic respiration, or fermentation (Ju et al., 2005). This indicated the possibility of constructing micro-ecosystems integrating anaerobic and aerobic niches because of the anaerobes and aerobes that are able to survive in one reactor.

Shen and Guiot (1996) investigated the impact of influent dissolved oxygen (DO) on the characteristics of anaerobic granules at various DO concentrations (0.5-8.1 ppm) in 1-L and 5-L laboratory-scale modified upflow anaerobic sludge bed (UASB) reactors. These results show that the anaerobic/aerobic coupled reactor can be successfully operated under O_2-limited conditions and could function as an ideal engineered ecosystem that integrates aerobic and anaerobic niches. Compared to the conventional aerobic process, micro-aerobic systems are more energy-efficient requiring less energy for aeration. If such a treatment process could be induced to concurrent removal of organic substances and nutrients, an important step in wastewater treatment appears to be possible. A novel, micro-aerobic bioreactor characterized by a lower DO level (0.4-0.8 mg/L) and higher OLR (3.3-3.5 kg COD/$m^3 \cdot$d) than those in conventional activated sludge reactor is proposed here to achieve lower energy consumption and lower capital costs.

Cloning and sequencing of PCR-amplified 16S rRNA gene fragments have been successfully applied for the analysis of bacterial community structure in a wide range of environmental samples (Calheiros et al., 2010). In this study, this approach was used to determine bacterial community structure and biodiversity in the MABR.

Research into the operational characteristics of waste-water treatment facilities, operating at low DO levels, is of great importance in terms of energy saving and the development of novel processes. More work should be done to obtain a clear understanding for this system. The main objective of this research is to investigate the impact of DO concentration on the performance of two activated sludge bed reactors treating high organic wastewater. Special attention was paid to the operating characteristics under limited aeration condition. It is expected that this study will provide some fundamental information for the recognition and application of micro-aerobic treatment processes.

MATERIALS AND METHODS

Synthetic wastewater composition and seed sludge

Glucose was used as the sole organic source of synthetic wastewater. The synthetic wastewater with the following composition was used: glucose, 3.5 g/L; $(NH_4)_2SO_4$, 0.7 g/L; KH_2PO_4, 0.2 g/L; NaCl, 0.1 g/L; $CaCl_2$, 0.03 g/L; $MgSO_4 \cdot 7H_2O$, 0.1 g/L; $NaHCO_3$, 0.2 g/L; tap water.

Reactor set-up and operation

We used two parallel identical laboratory-scale column-type continuous flow reactors comprising a plastic feed tank and a lucite aerated column. The two reactors were provided with a hopper bottom with the following dimensions: length of 90 cm, internal diameter of 10 cm and working volume of 6.3 L. The metering pump transferred wastewater from the feed tank to the bottom of the aerated column at a controlled rate. The effluent overflowed from the top of the aerated column and was subsequently discharged. Air was introduced via a porous stone diffuser at the bottom of the reactor. A heating rod was used to maintain the temperature of the reactor at 25±1°C. The scheme of the reactor is shown in Figure 1.

Table 1. Performance of the MABR and ABR at steady phase.

Parameter	COD (mg/l)			TN (mg/l)			TP (mg/l)		
	Inf.	Eff.	Rem.	Inf.	Eff.	Rem.	Inf.	Eff.	Rem.
MABR	3427±58	291±56	90±3	167±5	46±6	73±3	44±2	17±2	62±4
ABR	3427±58	185±84	93±3	167±5	22±3	86±3	44±2	23±3	43±2

MABR, micro-aerobic bioreactor; ABR, aerobic bioreactor; COD, chemistry oxygen demand; TN, total nitrogen; TP, total phosphate; Inf., influent; Eff., effluent; Rem., removal rate.

A 2 g sample of activated sludge and soil was placed in the aerated column containing 6.3 L of synthetic wastewater. The mixture was incubated in batch mode for two days to obtain enough biomass prior to continuous operation. The synthetic wastewater was prepared daily, after which it was continuously pumped into the aeration column; water sampling and *situ* DO and pH measure-ments were conducted daily in the upper part of the reactor. The system had a hydraulic retention time (HRT) of 25 h and an organic loading rate (OLR) of 3.3-3.5 kg COD/ m^3·d. The DO level of the aeration column was controlled manually and maintained at 3-4 mg/L and 0.4-0.8 mg/L in the ABR and MABR, respectively. The pH was adjusted to 7.0±0.5 by adding 1 M $NaHCO_3$ or 1 M HCl.

Analytical methods

Chemistry oxygen demand (COD); total nitrogen (TN) and TP were measured according to standard methods (APHA, 2005). DO was determined by DO meter (HANNA HI 9145).pH was determined by pH meter (HANNA HI 8124). The average values and standard deviations of the consecutive measurement sets collected under the quasi-steady-state conditions were considered to represent the corresponding treatment and were individually compared with a one-way analysis of variance (ANOVA) using IBM SPSS statistics 20.0, P<0.05 was considered significant difference.

DNA extraction and PCR amplification

0.5 g of wet sludge was washed three times using phosphate-buffered saline (PBS, pH 7.0) , followed by centrifugation (5000 rpm) at 4°C, for 5 min. The genomic DNA was then extracted using the method of Tsai and Olson (1991). Approximately 10 µg DNA g^{-1} of wet sludge was obtained using electrophoresis on 1% (w/v) agarose gel and visually compared with a molecular mass ladder. To acquire suitable amplicons, 10-100-fold dilutions of crude DNA were used as templates for subsequent PCRs.

To minimize PCR bias, three separate reactions were run for each sample and pooled. PCR amplication of the 16S rRNA gene fragments was carried out using the forward primer 27f (5'- AGA GTT TGA TCC TGG CTC AG-3') and the reverse primer 1492r (5'-GGT TAC CTT GTT ACG ACT T-3'). The conditions for the PCR amplification were as follows: initial denaturation for 5 min at 95°C, followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 52°C, extension for 1.5 min at 72°C, and final extension for 8 min at 72°C. PCR products were purified using TaKaPa purification kits.

Cloning and sequencing of 16S rRNA gene

The cloning of amplified 16S rRNA gene fragments into the TA cloning vector PMD18-T was performed following the manufac-turer's instructions. Then, DH5α chemically competent *E. coli* was transformed with the plasmids according to manufacturer's instructions. Transformants were selected by blue-white selection on Luria-Bertani agar plates containing amplicillin (150 µg/mL). Cloned inserts were amplified from lysed colonies by PCR with plasmid-vector specific primers M13F (5'-GTA AAA CGA CGG CCA G-3') and M13R (5'-CAG GAA ACA GCT ATG AC-3'). Clones were sequenced by Beijing Sunbiotech Co.,Ltd.

Phylogenetic analysis

All the nucleotide sequences were searched against the Ribosomal Database Project (RDP, release 10.0) and the GenBank database using the BLAST program. Phylogenetic trees were constructed using MEGA version 4.0 by the neighbor-joining algorithm, and the Jukes-Cantor distance estimation method with bootstrap analyses for 1000 replicates was performed. The possible chimeras in the obtained sequences were checked using the BELLEROPHON prior to phylogenetic analysis (Huber et al., 2004).

Nucleotide sequence accession numbers

The particle 16S rRNA gene sequences that were determined have been deposited in the GenBank, nucleotide sequence databases under accession nos. JN620460-JN620494.

RESULTS

Operation performance of ABR and MABR

ABR and MABR were operated for 60 days. Table 1 presents the pollutant concentration in the influent, effluent and pollutant removal efficiency at the steady stage. These results indicate that although the DO con-centrations were very different in the ABR and MABR, both of these systems indicated good performance in terms of COD removal. The concentration of COD in the influent was 3427±58 mg/L, after 20 days of acclimation, and the effluent COD of the MABR and ABR stabilized at levels of 291±56 and 185±84 mg/L, respectively. The COD removal efficiency stabilized to levels of 93±3 and 90±3% respectively; both reactors had high COD removal efficiency and there was no significant difference (P=0.087>0.05).

Nutrients removal and conversion process

During the 60 days operating period, influent TN and TP concentrations were measured at 167±5 and 44±2 mg/L

respectively. After 22 days of acclimation, the effluent TN of ABR and MABR were 22±3 and 46±6 mg/L respectively, and the TN removal efficiency of the ABR (86±3%) was higher than that of the MABR(73±3%). At the steady stage, TP concentrations in the effluents of ABR and MABR were 23±3 and 17±2 mg/L, respectively and the TP removal efficiency of the MABR (62±4%) was thus significantly higher than that of ABR(43±2%) (P=0.00).

Diversity and phylogenetic analysis

To obtain more detailed information on microbial community in the MABR, partial 16S rDNA fragments were PCR-amplified from the extracted DNA using bacterial specific primers sets, 35 bacterial clones were obtained from MABR. The phylogenetic tree (Figure 2) indicated that bacteria in the MABR was highly diverse. Analyzed clones represented four different phyla of the domain bacteria. These belonged to the *Firmicutes* (54.3%), *Actinobacteria* (20%), *Proteobacteria* (17.1%) and *Bacteroidetes* (8.6%). In the phylum of *Firmicutes*, *Bacilli* (48.6%) was the most abundant class followed by *Clostridia* (5.7%), suggesting that the *Bacilli* was the dominant group in the bacterial population. All the clones in the class of *Bacilli* belong to the *Lactobacillales* order that comprise the lactic acid bacteria according to RDP analysis. Lactic acid bacteria are Gram-positive, facultative anaerobic organisms that can convert sugars to lactic acid. Clones of W4, W13, W36, W37, W40 and W54 clustered together and had the highest identity score of 99% with *Leuconostoc pseudomesenteroides*, a lactic acid bacteria isolated from Tibetan Qula cheese (Duan et al., 2008). The abundance of sequences related to lactic acid bacteria clearly indicates the glucose fermentation and facultative anaerobes might exist in the MABR. Clones of W12, W21, W47, W51, and W46 clustered together and had the highest identity with an uncultured bacterium clone PeH08 isolated from Midgut and Hindgut of the Humus-Feeding Larva of *Pachnoda ephippiata* (Egert et al., 2003). Clones of W3, W24, W42 had the highest identity score of less than 96% with uncultured bacterium clone isolated from gut. There were seven clones clustered together in *Actinobacteria*, six of them were classified into *Bifidobacterium* spp. andshowed a relationship with *Bifidobacterium minimum*, *Bifidobacterium psychraerophilum* and *Bifidobacterium subtile*. Bifidobacteria are anaerobic or facultatively anaerobic bacteria that are typically found in the intestinal tract of humans and animals, also from environmental materials such as sewage and anaerobic digester (Watanabe et al., 2009). Wertz and Breznak (2007) reported that there were low concentrations of DO in the gut and most of the microbes isolated from gut were microaerophile. This indicated that there might be anaerobes

and microaerophile that existed in the MABR. According to RDP analysis, sequences of the phylum *Proteobacteria* were classified into *Alphaproteobacteria* (5.7%) and *Gammaproteobacteria* (11.4%). All the sequences in the cluster of *Alphaproteobacteria* and *Gammaproteobacteria* were classified in the family of *Rhodobacterales* and *Enterobacteriaceae* respectively. Members of the *Enterobacteriaceae* are facultative anae-robic organisms fermenting sugars to lactate and various other end products. The phylogenetic analysis results show that anaerobes and microaerophiles were able to survive in the MABR. The coexistence of facultative anaerobes and microaerophile suggests that micro-aerobic and anaerobic niches might coexist in the MABR and that the MABR might function in a similar way to an aerobic/-anaerobic sequencing bioreactor.

DISCUSSION

He et al. (2009) reported that organic substances could be significantly biodegraded in the reactor at DO in the range of 0.8-5 mg/L. Peng et al. (2001) reported that under the condition of OLR of 2.4 kg COD /m^3·d, and DO of 0.8 mg/L, the COD removal efficiency reached to 95%. In the present study, both reactors had high COD removal efficiencies; the results were consistent with the findings listed above. ABR and MABR have similar COD removal efficiency, however, compared with the ABR (48 L/h), the air flow rate of the MABR was 21 L/h, resulting in 56% saving in aeration costs. From the operational point of costs, operation at low DO reduces energy consumption.

In the last decades, enhanced biological phosphorus removal (EBPR) in activated sludge systems has become a widely applied wastewater treatment process for the removal of P without the use of chemical precipitation. EBPR can be achieved through the activated sludge process by re-circulating sludge through anaerobic and aerobic conditions (Oehmen et al., 2007). Ju et al. (2005) reported that under micro-aerobic conditions, the organisms might simultaneously perform aerobic and anaerobic respiration, or the fermentation and the aerobic and anaerobic niches might coexist, since the anaerobes and aerobes are able to survive in such systems. This means that the MABR might function like the aerobic/anaerobic sequential process and has the higher P removal efficiency than ABR. In our study, the air flow rate of the MABR was only 21 L/h, which was much less than ABR (48 L/h). Brdjanovic et al. (1998) reported that excessive aeration clearly negatively affects the biological phosphorous removal (BPR) processes. Our result is consistent with the finding listed above. There were a group of microorganisms that are largely responsible for P removal in the EBPR, which are known as the poly-phosphate accumulating organisms (PAOs). The most well known PAO group was *Candidatus* Accumulibacter
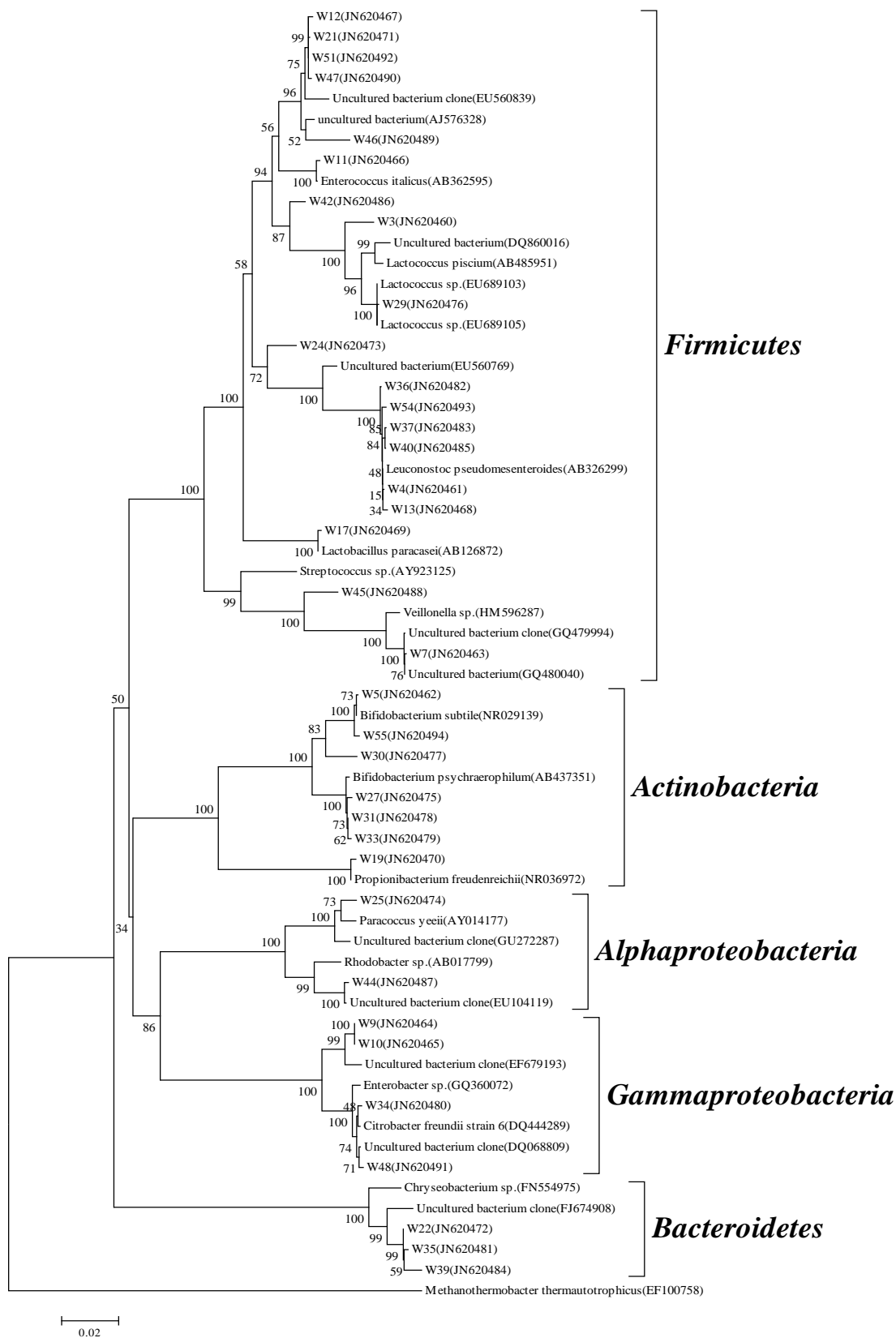
Figure 2. Phylogenetic trees constructed by NJ method and showing the phylogenetic positions of all the clones. Bootstrap replication was 1000. Scale bar estimated difference in nucleotide sequences positions.

phosphatis (*Accumulibacter*), closely related to *Rhodocyclus* in the *Betaproteobacteria*. Results illustrated in Figure 2 indicate that none of the sequences detected from the MABR belonged to the known PAOs. There were numerous factors affecting the proliferation of PAOs, among them, the microbial competition of PAOs and glycogen accumulating organisms (GAOs) was the focus of many studies. The proliferation of GAOs, which compete with PAOs for the carbon sources without contributing to the EBPR process, results in reduced biological P removal efficiency (Oehmen et al., 2010). Application of different carbon source and ratios of organic carbon to P in the influent have been shown to have significant impacts on the competition between the PAOs and GAOs (Ahn and Speece, 2006). Numerous studies have indicated that COD/P ratio (for example >50 mgCOD/mgP) in the wastewater feed tends to favor the growth of GAOs instead of PAOs. A low COD/P ratio (10-20 mgCOD/mgP) should be more favorable to the growth of PAOs (Mino et al., 1998). Beside, Mino et al. (1998) also noted that competition for the carbon source was the deciding factor affecting the predominance of particular organism. Zengin et al. (2010) reported that continued glucose feeding favored preferential growth of GAOs over PAOs through direct glycogen metabolism. In this research, glucose was the sole carbon source and the COD/P ratio in the influent was as high as 74-85 mg COD/mgP, which was not suitable for the growth of PAOs. This might be the reason why no known PAOs were detected from the MABR. However, there were 28.6% of the sequences belonging to the cluster of *Actinobacteria*, *Flavobacteria*, which were commonly detected in EBPR processes (Zengin et al., 2011). This indicated that MABR might function like a conventional EBPR process.

EBPR-based treatment can be extremely efficient, but its requirement for anaerobic pretreatment zones may be problematic when retrofitting existing activated sludge installations. Moreover, the EBPR process may display inconsistencies in performance, since for optimal results, relatively high concentrations of VFAs need to be present in the influent wastewater (Mullan et al., 2005). For these reasons, the availability of effective yet economical P removal strategies is thus of great importance to the water industry. Mullan et al. (2005) reported that a single-stage aerobic P removal system might have advantages over conventional EBPR technology in terms of (a) rate of throughput, (b) tolerance of high nitrate levels (since the anaerobic phase of EBPR is sensitive to nitrate), (c) reduced dependence upon wastewater strength, and (d) operation at low VFA concentrations.

In our research, the P removal efficiency of the MABR was higher than that of the ABR, thus indicating that the EBPR process could function in a single-stage activated sludge bioreactor operating under microaerobic conditions. Expect for P, the micro-aerobic treatment system

could be better alternative for treatment of many pollutants requiring aerobic/anaerobic sequential treatment. Meanwhile, the MABR used less oxygen which means energy saving and lower initial and operating costs than conventional EBPR process.

According to our knowledge, researches regarding micro-aerobic technology were focused on performance (Chu et al., 2006; Díaz et al., 2010). The present study may therefore represent the first report of cultivation-independent molecular approaches for elucidating the phylogenetic composition, and the diversity of the microbial communities in an microaerobic bioreactor. Microaerobic technology required advances concerning not only the identity and biodiversity of the micro-organisms involved in the systems, but also their biochemistry and metabolism. Beyond the taxonomy of organisms, which was now routinely studied using rDNA and rRNA-based methods, the next challenge is to obtain more information about the genetics and gene expression of the microaerobic-related enzymes.

Conclusions

The results of this research indicate that it is possible to remove organic substances and phosphorous simultaneously in a single stage activated sludge bioreactor under oxygen-limited conditions. The findings are important in terms of recognizing and supplementing the EBPR process.

Operation at low DO means energy saving, as well as lower initial costs and operating costs. Phylogenetic analysis showed that anaerobes and microerophile were able to survive in the MABR, so the micro-aerobic treatment system might be a better alternative to the aerobic/anaerobic sequential processes. More work is necessary to understand the fundamental biochemical and micro-biological mechanisms of micro-aerobic treatment systems.

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Full Length Research Paper

Effects of aromatic amino acids, phenylacetate and phenylpropionate on fermentation of xylan by species of rumen anaerobic bacteria

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This study determined the effects of aromatic amino acids (AA) and phenyl acids (phenylacetic acid and phenylpropionic acid) on fermentation of xylan by selected rumen bacteria. The ruminal bacteria were grown in a defined medium containing oat spelts xylan as the sole energy source, plus one of the following N sources: ammonia (no AA); ammonia plus a complete mixture of 20 AA commonly found in protein (CAA); ammonia plus complete AA mixture minus aromatic AA (MAR); ammonia plus phenyl acids (no AA+PA); ammonia plus complete AA mixture without aromatic AA plus phenyl acids (MAR+PA). There was varied growth response, with *Fibrobacter succinogenes* S85, *Ruminococcus flavefaciens* FD1, and *Butyrivibrio fibrisolvens* JW11 not being able to grow. The supply of CAA mixture significantly ($P<0.05$) increased acetate production in all species, in comparison with no AA treatment, by 54, 30, 97 and 33% in *Prevotella bryantii* B_14, *Ruminococcus albus* SY3, *R. flavefaciens* 17 and *Pseudobutyrivibrio xylanivorans* JK170 cultures, respectively. The addition of phenyl acids significantly ($P<0.05$) increased acetate yields by 34, 8 and 5% in *P. bryantii* B_14, *R. albus* SY3 and *P. xylanivorans* JK170 cultures, respectively. Sugar utilization by all the species significantly ($P<0.05$) increased by 42 and 77% when CAA mixture was provided. The deletion of aromatic AA from the complete AA mixture caused a significant ($P<0.05$) reduction in sugar utilization in *P. bryantii* B_14 and *P. xylanivorans* JK170 by 25 and 10%, respectively. Phenyl acids addition increased sugar utilization by 4.8 and 5.1% in *R. albus* SY3 and *R. flavefaciens* 17, respectively. The supply of CAA mixture significantly ($P<0.05$) increased microbial yield in *P. bryantii* B_14, *R. albus* SY3, *R. flavefaciens* 17 and *P. xylanivorans* JK170 species. It is concluded that supplementation of complete mixture of AA is beneficial in stimulating bacterial growth in the rumen.

Key words: Aromatic amino acids, phenylacetic acid, phenylpropionic acid, rumen bacteria, xylan.

INTRODUCTION

Complete mixtures of all amino acids (AA) stimulate the rate of fermentation and the microbial growth yield (Cotta and Russell, 1982; Chen et al., 1987; Cruz Soto et al., 1993). However, aromatic AA have been known to limit the growth of microbes and xylan fermentation by mixed rumen micro-organisms (Kajikawa et al., 2002; Atasoglu et al., 2003; Guliye et al., 2005). Phenylalanine, one of

the aromatic AA, has been suggested to be essential for the growth of cellulolytic bacterial species, including *Ruminococcus* (Allison 1965) and *Fibrobacter succinogenes* (Allison, 1965; Atasoglu et al., 2001). Fungal strains of *Neocallimastix* spp., provided with cellobiose as energy source, also showed reduced growth when aromatic AA were omitted from a complete

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mixture of 18 AA (Onoda et al., 1996), and achieved maximum growth when a complete mixture were supplied (Orpin and Greenwood, 1986; Onoda et al., 1996; Guliye and Wallace, 2007).

The precursors for the synthesis of aromatic AA, phenylacetic acid (PAA) and phenylpropionic acid (PPA), can be beneficial to mixed populations of rumen bacteria and protozoa (Scott et al., 1964; Kristensen, 1974; Amin and Onodera, 1997). The phenyl acids (PAA and PPA) have also been reported to stimulate growth and to improve cellulose degradation by *Ruminococcus albus* (Allison, 1965; Hungate and Stack, 1982; Stack et al., 1983; Stack and Cotta, 1986; Morrison et al., 1990).

Fibrous plant roughages, a significant portion being hemicellulose, form the bulk of ruminant livestock diets. Xylan is a predominant polymer in hemicellulose. Much of xylan degradation occurs in the rumen and is carried out by a number of ruminal organisms, including bacteria. The key rumen bacteria involved in xylan degradation include *Butyrivibrio fibrisolvens* and *Prevotella ruminicola* (Dehority, 2003), *Prevotella bryantii* (Miyazaki et al., 1997), *Pseudobutyrivibrio xylanivorans* (Kopecny et al., 2003), and the cellulolytic species *Fibrobacter succinogenes*, *R. albus*, and *R. flavefaciens* (Hespell and Whitehead, 1990). Therefore, understanding the AA and phenyl acids effects on rumen bacteria may be important to ruminal xylan fermentation efficiency. However, there are limited pure culture data published on the possible effect(s) of aromatic AA, as a group, on xylan utilization and growth of the main xylanolytic organisms in the rumen. The aim of the present study was to determine the effects of aromatic AA or their derivative phenyl acids (PA) on the fermentation of xylan by selected rumen anaerobic bacteria.

MATERIALS AND METHODS

Organisms

The rumen bacteria used in this study were obtained from the culture collection maintained at the Rowett Research Institute (currently Rowett Institute of Nutrition and Health**)**, Aberdeen (UK), and were selected based on their presumed ability to degrade and utilize xylan (oat spelts xylan**)**, the substrate used as energy source. Therefore, the following organisms were used: *F. succinogenes* S85, *R. flavefaciens* 17, *R. flavefaciens* FD1, *R. albus* SY3, *B. fibrisolvens* JW11, *Pseudobutyrivibrio. xylanivorans* JK170 and *P. bryantii* B$_1$4.

Media and growth conditions

The organisms, obtained from stock cultures, were initially grown overnight under anaerobic conditions at 39°C in 10 ml of M2 medium (Hobson, 1969). The organisms were then inoculated (5%, vol/vol) into 100 ml Wheaton bottles containing the defined medium of Hungate and Stack (1982), modified to include 0.3% (w∕v) oat spelts xylan (OSX) (Sigma, Steinheim-Germany) as the sole energy source, to enable them adapt to the xylan. The cultures were then grown overnight at 39°C and checked for species conformation

through gram staining. These fresh cultures were then used to inoculate (5%, vol/vol) triplicate Wheaton bottles containing 100 ml of fresh modified Hungate and Stack medium, with various additions according to treatment specifications. The wheaton bottles were then placed in a shaking water bath at 39°C and the cultures were incubated for up to 36 h.

Treatments

There were five treatments: ammonia only (no AA); ammonia plus complete mixture of 20 AA commonly found in protein (CAA); ammonia plus the complete AA mixture but with aromatic AA omitted (MAR); ammonia plus phenyl acids (no AA+PA); ammonia plus the complete AA mixture with aromatic AA omitted, plus phenyl acids (MAR+PA). The concentration of ammonia, supplied in form of NH_4Cl to all treatments, was 10 mmol L^{-1}. The AAs were added to a final concentration of 0.25 g L^{-1} for each AA. The PAA and PPA were added, where applicable, to a final concentration of 10 and 25 mmol L^{-1}, respectively (Stack and Cotta, 1986; Stack et al., 1983). Treatments where PAA and PPA were not supplemented had the same volume of distilled water added instead. The N sources and phenyl acids were added to the media during preparation.

Experimental measurements

The Wheaton bottles were periodically shaken and samples obtained anaerobically via the septum using sterile needles and syringes at the start (10 ml), during (2 ml, at various time points) and at the end of the experiment (10 ml), when stationary phase had been reached. Two millilitres of the collected samples were solubilized in 0.5 mol l^{-1} NaOH and used to determine protein using the Bradford assay (Bradford, 1976). The remainder of the 0 h and stationary phase samples (18 or 24 h) were kept frozen and used later for the determination of volatile fatty acids (VFA), ammonia, sugar utilization and total cell N (TCN) as described by Guliye et al. (2005).

Calculations and statistical analysis

The results are all means derived from the analysis of triplicate cultures. Net values were calculated as 18 or 24 h concentration - 0 h concentration for VFA, ammonia, sugar utilization and TCN. Microbial yield (expressed as g N kg^{-1} sugar utilised) was calculated from the net microbial N synthesis (TCN) divided by the net sugar utilization. The data were analysed by ANOVA, with N source (no AA, CAA, MAR) and phenyl acids addition (no AA+PA and MAR+PA) as treatment effects, using Genstat program release 6.1 (Lawes Agricultural Trust, Rothamsted Herts, UK). Multiple mean comparisons were done using Fisher's comparison.

RESULTS

Bacterial growth

All species from the stock cultures grew well in the M2 medium. However, when transferred into the initial modified Hungate and Stack medium, which was used as inoculum, not all cultures grew actively. While *P. bryantii* B$_1$4, *R. albus* SY3, *R. flavefaciens* 17 and *P. xylanivorans* JK170 strains grew well, *F. succinogenes* S85, *R. flavefaciens* FD1 and *B. fibrisolvens* JW11 were less

Figure 1. Growth of ruminal bacteria on oat spelts xylan in the presence of: ammonia only with no amino acids - AA (◆), complete AA mixture - CAA (■), complete AA mixture minus aromatic AA - MAR (▲), ammonia only plus phenyl acids - no AA+PA (✳), and complete AA mixture minus aromatic AA plus phenyl acids - MAR+PA (●). Data points represent mean values from triplicate cultures.

active in their growth. When transferred into the final growth medium of modified Hungate and Stack, *P. bryantii* $B_1$4, *R. albus* SY3, *R. flavefaciens* 17 and *P. xylanivorans* JK170 strains all grew, the first two strains having reached stationary phase at 18 h while the latter two reached at 24 h (Figure 1). *P. bryantii* $B_1$4 showed the highest growth, closely followed by *R. albus* SY3. However, *F. succinogenes* S85, *R. flavefaciens* FD1, and *B. fibrisolvens* JW11 failed to grow in the modified Hungate and Stack final growth medium (Figure 2). The growth observed earlier with the modified Hungate and Stack inoculum media may have been due to nutrients, especially energy source, transferred in the M2 medium.

Volatile fatty acids (VFA) and ammonia

Acetate was the major VFA produced by all the species that were able to grow (Table 1). In addition, *P.*

xylanivorans JK170 also produced significant amounts of butyrate. None of the species produced propionate or caproate irrespective of treatments.

The supply of complete AA mixture (CAA treatment) significantly ($P<0.05$) increased acetate production in all the species, in comparison with no AA treatment, by 54, 30, 97 and 33% in *P. bryantii* $B_1$4, *R. albus* SY3, *R. flavefaciens* 17 and *P. xylanivorans* JK170 cultures, respectively. While valerate was not significantly influenced by the addition of complete mixtures of AA in *R. albus* SY3 and *R. flavefaciens* 17, there was 13 fold increase in iso-butyrate by *P. bryantii* $B_1$4 (Table 1). Also, butyrate production by *P. xylanivorans* JK170 increased significantly ($P<0.05$) by fivefold in complete AA mixtures (Table 1).

The deletion of aromatic AA from a complete mixture (MAR treatment) did not influence acetate yields in *R. flavefaciens* 17 and *P. xylanivorans* JK170, however, there was 24 and 5% decline ($P<0.05$) observed in *P. bryantii* $B_1$4

Figure 2. *Fibrobacter succinogenes* S85 / *Ruminococcus flavefaciens* FD1 / *Butyrivibrio fibrisolvens* JW11
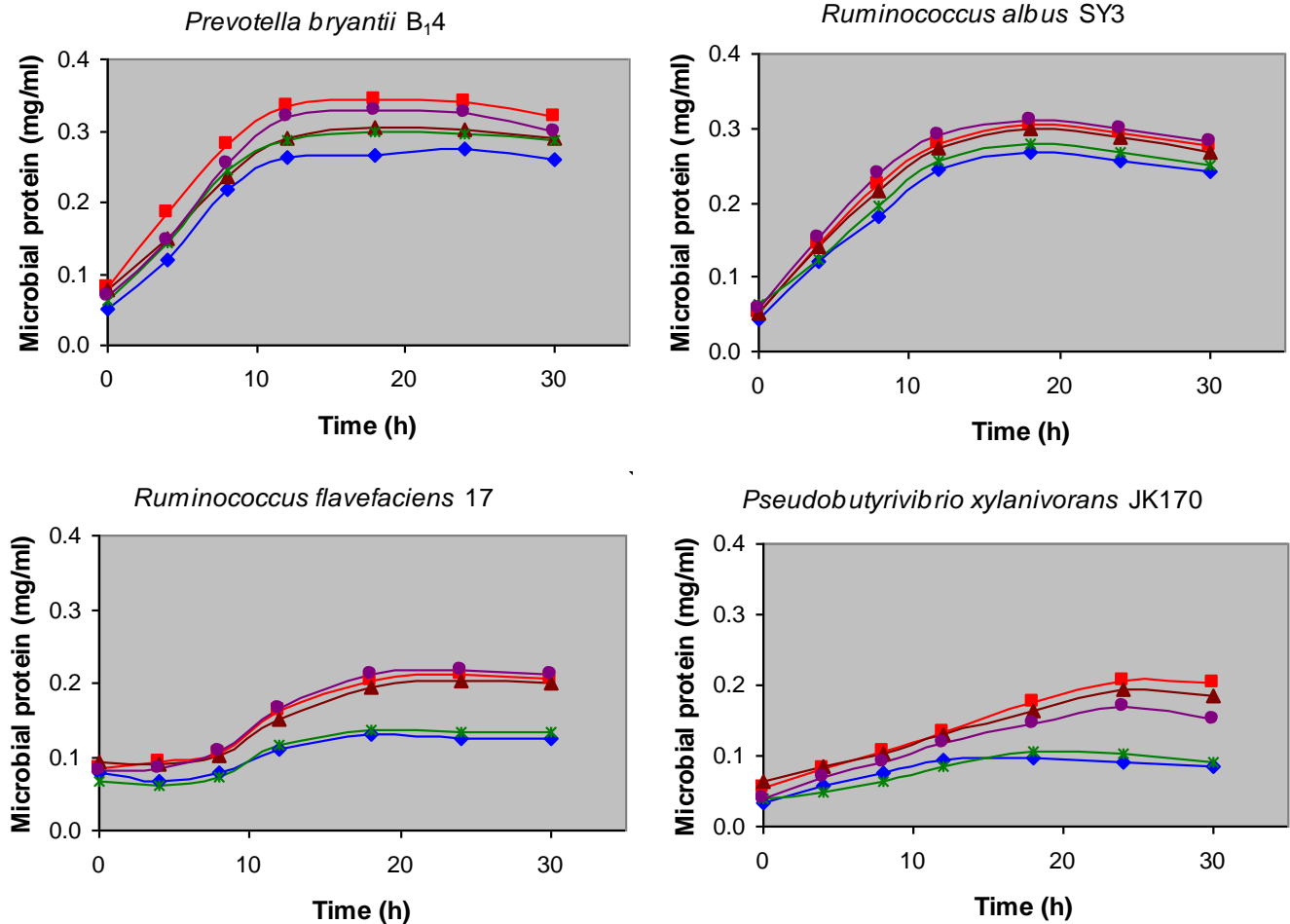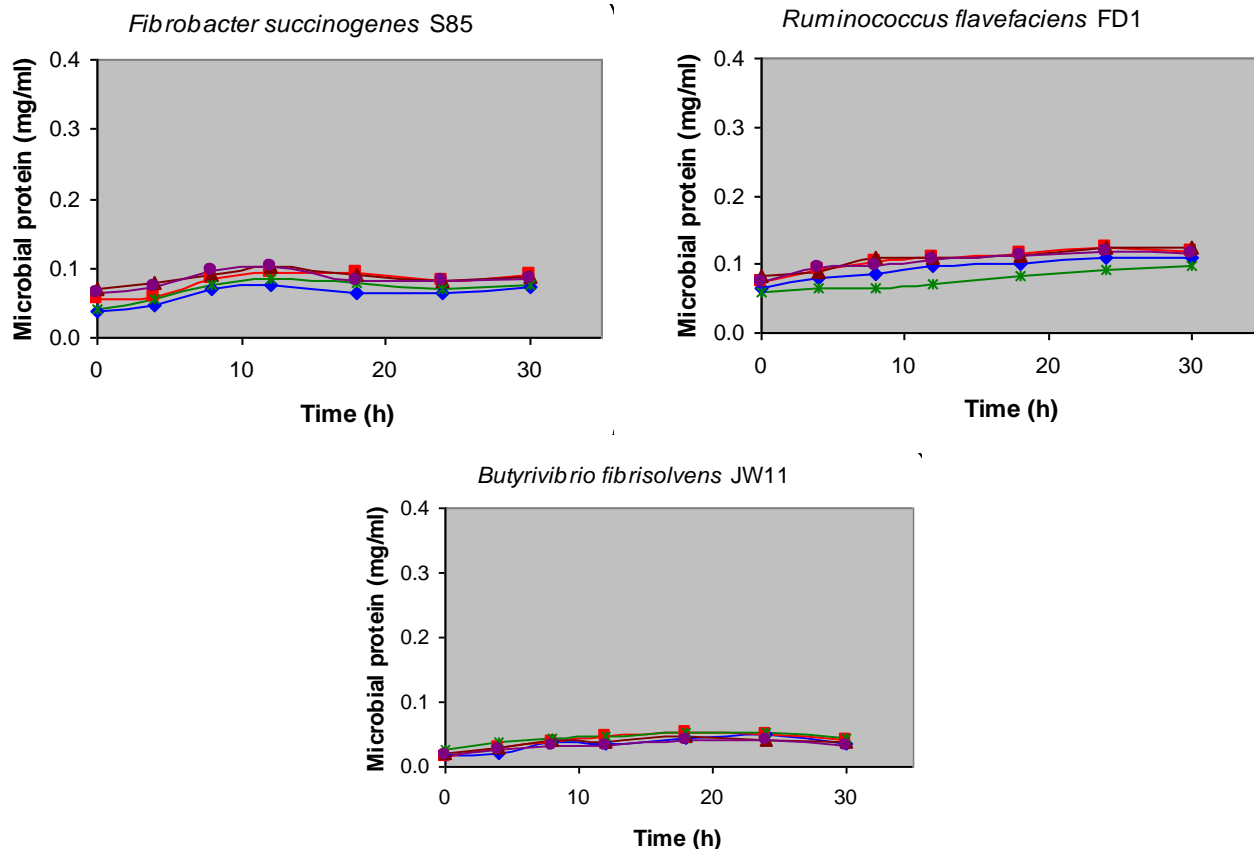

Figure 2. Growth of ruminal bacteria on oat spelts xylan in the presence of: ammonia only with no amino acids - AA (♦), complete AA mixture - CAA (■), complete AA mixture minus aromatic AA - MAR (▲), ammonia only plus phenyl acids - no AA+PA (✳), and complete AA mixture minus aromatic AA plus phenyl acids - MAR+PA (●). Data points represent mean values from triplicate cultures.

Table 1. Effects of aromatic AA and phenyl acids (PA) on volatile fatty acids (VFA) production by ruminal bacteria fermenting xylan.

Bacterial species	VFA (mmol l^{-1})	no AA	CAA	MAR	no AA +PA	MAR +PA	SEM
Prevotella	Acetate	3.11^a	4.78^b	3.64^c	3.32^d	4.72^b	0.06
Bryantii B$_1$4	iso-butyrate	0.01^a	0.13^b	0.11^b	0.00^a	0.06^c	0.01
Ruminococcus	Acetate	4.83^a	6.25^b	5.92^c	5.01^a	6.39^b	0.04
Albus SY3	Valerate	0.00	0.02	0.01	0.01	0.01	0.01
Ruminococcus	Acetate	2.35^a	4.64^b	4.58^b	2.72^a	4.59^b	0.25
Flavefaciens 17	Valerate	0.01^a	0.04^a	0.01^a	0.04^a	0.12^b	0.01
Pseudobutyrivibrio	Acetate	3.20^a	4.25^{b,d}	4.17^b	3.68^c	4.37^d	0.05
Xylanivorans JK170	Butyrate	0.83^a	3.88^b	3.41^c	0.96^a	3.25^c	0.07

Treatments were: no AA = ammonia only, CAA = ammonia + complete mixture of 20 amino acids, MAR = ammonia + complete mixture minus aromatic amino acids, no AA+PA = ammonia only plus phenyl acids, and MAR+PA = ammonia + complete mixture minus aromatic amino acids plus phenyl acids. Phenyl acids (PA) were phenylacetic acid (10 mmol L^{-1}) and phenylpropionic acid (25 mmol L^{-1}). Amino acids were added at 0.25 g L^{-1} for each amino acid. Xylan (sugar) was added at 3 g L^{-1}. Values are means calculated from triplicate cultures. Means in the same row with different superscripts differ (P<0.05). Propionic, butyric (except for P. xylanivorans JK170) and caproic acids were not detected in all treatments. Valeric, iso-butyric (except for P. bryantii B14) and iso-valeric acids appeared as traces.

and *R. albus* SY3, respectively. The addition of phenyl acids, PAA and PPA, to ammonia (no AA treatment) significantly (P<0.05) increased acetate production by 7 and 15% increase in *P. bryantii* B$_1$4 and *P. xylanivorans*

Table 2. Effects of aromatic AA and phenyl acids (PA) on ammonia (mg l^{-1}) production by ruminal bacteria fermenting xylan.

Organism	Treatment					
	no AA	CAA	MAR	no AA+PA	MAR+PA	SEM
Prevotella bryantii $B_1$4	-24.3[a]	85.5[b]	73.6[c]	-31.1[a]	60.2[d]	2.22
Ruminococcus albus SY3	-43.9[a]	54.7[b]	46.2[c]	-48.0[a]	36.3[d]	1.19
Ruminococcus flavefaciens 17	-24.4[a]	35.4[b]	29.8[c]	-26.5[a]	33.9[b]	0.56
Pseudobutyrivibrio xylanivorans JK170	-19.9[a]	32.9[b]	31.1[b,c]	-21.4[a]	29.1[c]	0.54

Treatments were: no AA = ammonia only, CAA = ammonia + complete mixture of 20 amino acids, MAR = ammonia + complete mixture minus aromatic amino acids, no AA+PA = ammonia only plus phenyl acids, and MAR+PA = ammonia + complete mixture minus aromatic amino acids plus phenyl acids. Phenyl acids (PA) were phenylacetic acid (10 mmol l^{-1}) and phenylpropionic acid (25 mmol L^{-1}). Amino acids were added at 0.25 g L^{-1} for each amino acid. Xylan (sugar) was added at 3 g l^{-1}. Values are means calculated from triplicate cultures. Means in the same row with different superscripts differ (P<0.05).

JK170, respectively (Table 1). Also, phenyl acids addition to a complete mixture of AA with aromatic AA omitted (MAR) (MAR) significantly (P<0.05) increased acetate yields by 34, 8 and 5% in *P. bryantii* $B_1$4, *R. albus* SY3 and *P. xylanivorans* JK170 cultures, respectively. In all species, acetate production was not significantly different between CAA and MAR+PA treatments.

Although butyrate production decreased (P<0.05) by about 12% as a result of the deletion of aromatic AA from a complete AA mixture in *P. xylanivorans* JK170, the addition of phenyl acids to a medium that contained complete AA mixtures with aromatic AA omitted (MAR+PA treatment) did not significantly influence butyrate production (Table 1). Butyrate yields by *P. xylanivorans* JK170 was significantly lower in MAR+PA treatment, compared to CAA, despite the addition of phenyl acids. Valerate yields increased (P<0.05) over ten fold in *R. flavefaciens* 17 due to addition of phenyl acids, in comparison with MAR treatment (Table 1), however, it was not influenced by the deletion of aromatic AA from a complete AA mixture in *R. albus* SY3 and *R. flavefaciens* 17. The addition of AA, whether complete or with aromatic AA omitted, significantly (P<0.05) increased iso-butyrate yields by *P. Bryantii* $B_1$4, in comparison with ammonia only (no AA) treatment (Table 1). However, compared to MAR treatment, there was almost 45% decline in iso-butyrate production by *P. bryantii* $B_1$4 when phenyl acids were provided (MAR+PA treatment).

The addition of AA, whether the complete mixture or minus aromatic AA, resulted in increased ammonia yields by all the organisms, the increase being highest in the treatment where complete mixtures of AA (CAA) were supplemented (Table 2). The yields of ammonia produced declined in all the species when aromatic AAs were omitted from a complete mixture of AA. The decrease, significant at P<0.05, was 14, 16 and 16% for *P. bryantii* $B_1$4, *R. albus* SY3 and *R. flavefaciens* 17, respectively.

Phenyl acids added to a medium that contained only ammonia as sole N source had no significant influence on ammonia production. Conversely, the addition of phenyl acids to complete AA mixtures with aromatic AA omitted (MAR+PA treatment) led to a signi-ficant (P<0.05) decrease in ammonia in most species, in comparison with MAR treatment. The decrease was 18, 21 and 6% for *P. bryantii* $B_1$4, *R. albus* SY3, and *P. xylanivorans* JK170, respectively. However, there was a 14% increase in ammonia production by *R. flavefaciens* 17. The net ammonia in the treatments where AA were not provided (no AA and no AA+PA) had negative values (Table 2), since the microorganisms utilised the ammonia in the medium, which was the only source of N provided.

Sugar utilization

The utilization of xylan (OSX), as the sole energy source, by the bacterial species is presented in Table 3. The amount of sugar (xylan) utilised was greatest for *P. bryantii* $B_1$4 and *R. albus* SY3. The addition of AA, whether the complete mixture or minus aromatic AA, stimulated sugar utilization. Compared with no AA treatment, sugar utilization by all the species significantly (P<0.05) increased by between 42 and 77% when a complete mixture of AA was provided. The deletion of aromatic AA from the complete AA mixture caused a significant (P<0.05) reduction in sugar utilization only in *P. bryantii* $B_1$4 and *P. xylanivorans* JK170 by 25 and 10%, respectively).

The addition of phenyl acids to a medium that contained ammonia as the only source of N (no AA *vs* no AA+PA treatments) did not significantly increase sugar (xylan) utilization, except in *P. bryantii* $B_1$4 where a 25% increase (P<0.05) was observed (Table 3). However, compared to the MAR treatment (that is, complete AA mixture with aromatic AA omitted), addition of phenyl acids significantly (P<0.05) increased sugar utilization only in *P. bryantii* $B_1$4 and *R. albus* SY3 by 20 and 13%, respectively. Compared to CAA treatment, the addition of phenyl acids to a medium that had complete AA mixture with aromatic AA omitted (MAR treatment) showed increased utilization of sugar by 4.8% and 5.1% in *R. albus* SY3 and *R. flavefaciens* 17, respectively, which was however, significant (P<0.05) only for the former.

Table 3. Effects of aromatic AA and phenyl acids (PA) on xylan utilization by species of rumen bacteria.

Organism	Sugar utilised	Treatments					SEM
		no AA	CAA	MAR	no AA +PA[1]	MAR +PA[1]	
Prevotella	g/L	1.21^a	1.95^b	1.50^c	1.51^c	1.79^d	0.03
Bryantii $B_1 4$	%	40	65	50	50	60	
Ruminococcus	g/L	1.25^a	1.68^b	1.60^b	1.32^a	1.76^c	0.03
Albus SY3	%	42	56	53	44	59	
Ruminococcus	g/L	0.79^a	$1.17^{b,c}$	1.11^b	0.81^a	1.23^b	0.02
Flavefaciens 17	%	26	39	37	27	41	
Pseudobutyrivibrio	g/L	0.62^a	1.07^b	1.00^c	0.65^a	0.95^c	0.02
Xylanivorans JK170	%	21	36	33	22	32	

Treatments were: no AA=ammonia only, CAA= ammonia + complete mixture of 20 amino acids, MAR = ammonia + complete mixture minus aromatic amino acids. Phenyl acids (PA) were phenylacetic acid (10 mmol L^{-1}) and phenylpropionic acid (25 mmol L^{-1}). Amino acids were added at 0.25 g L^{-1} for each amino acid. Xylan (sugar) was added at 3 g L^{-1}. Values are means calculated from triplicate cultures. Means in the same row with different superscripts differ ($P<0.05$).

Microbial yield

Microbial yield (g TCN kg^{-1} sugar utilised), calculated from the initial and final concentrations of cell protein and sugar (xylan), is presented in Figure 3. The yields recorded in the present study ranged between 27 and 31 for *P. bryantii* $B_1 4$; between 27 to about 30 for *R. albus* SY3; between 21 and 28 for *R. flavefaciens* 17; and between 17 and 27 for *B. fibrisolvens* 170 (Figure 3).

The addition of a complete AA mixture to a medium that had only ammonia as N source (no AA *vs* CAA treatments) significantly ($P<0.05$) increased microbial yield from 27.4 to 32.0, 27.2 to 29.3, 20.8 to 27.9, and 16.9 to 27.4 g kg^{-1} for *P. bryantii* $B_1 4$, *R. albus* SY3, *R. flavefaciens* 17 and *P. xylanivorans* JK170 species, respectively. The deletion of aromatic AA from a complete AA mixture (MAR treatment) did not significantly decrease microbial yields in all species, except for *P. bryantii* $B_1 4$ which showed about 7% decrease in yields.

The addition of phenyl acids to a medium that contained only ammonia as N source (no AA *vs* no AA+PA treatments) did not significantly increase microbial yield in all the species, except for *P. bryantii* $B_1 4$ which showed 8% increase in yield from 27.4 to 29.7 g kg^{-1} (Figure 3). Microbial yields in treatments where phenyl acids were added to a medium that had complete AA mixture with aromatic AA omitted (MAR+PA treatment) were 30.9, 29.6, 28.0 and 26.8 for *P. bryantii* $B_1 4$, *R. albus* SY3, *R. flavefaciens* 17 and *P. xylanivorans* JK170, respectively, none of which was significantly different from MAR treatment (Figure 3).

DISCUSSION

The effects of aromatic AA and phenyl acids on some species of rumen bacteria provided with Xylan, in form of OSX, as the only energy source were assessed. Due to the complex chemical nature of xylan, a variety of ruminal microorganisms are needed to hydrolyze and metabolize these polysaccharides (Hespell and Whitehead, 1990), although there are few exceptions. However, differences have been reported between strains and species of ruminal bacteria in their abilities to digest isolated xylans or xylans that are present in intact forages (Dehority, 2003; Hespell and Whitehead, 1990).

Growth and sugar utilization

There were varied growth responses in the present study where some rumen bacteria were able to grow, albeit to different extents, while others could not (Figures 1 and 2), perhaps due to inability to utilise xylan. Previous experiment by Hespell et al. (1987) comparing the abilities of various ruminal bacteria to degrade xylans in a Tryticase/yeast extract containing medium observed that *B. fibrisolvens* strain ARD22a could not grow at all on xylan substrates, while strains H17c and S2 showed little growth. They also noted that although *R. albus* 7, *R. flavefaciens* (C94 and FD1) and *F. succinogenes* S85 appeared to ferment xylan to some extent, only small amounts were utilized, and therefore little or no growth occurred. Studies by Jun et al. (2003) and Yoshida et al. (2010) clearly indicate that *F. succinogenes* S85 has xylanase genes that enable production of enzymes that can degrade xylan.

Earlier studies by Dehority (1965) showed that although major cellulolytic ruminal bacteria (that is, *F. succinogenes*, *R. albus* and *R. flavefaciens*) often can degrade hemicelluloses, only a few strains are capable of growing on these substrates. Some *R. flavefaciens* strains have been shown to utilise more than 50% of the pentose sugar present in bromegrass cell walls, but others showed little or no detectable pentose utilization (Dehority et al., 1967; Dehority, 2003). The differences in sugar
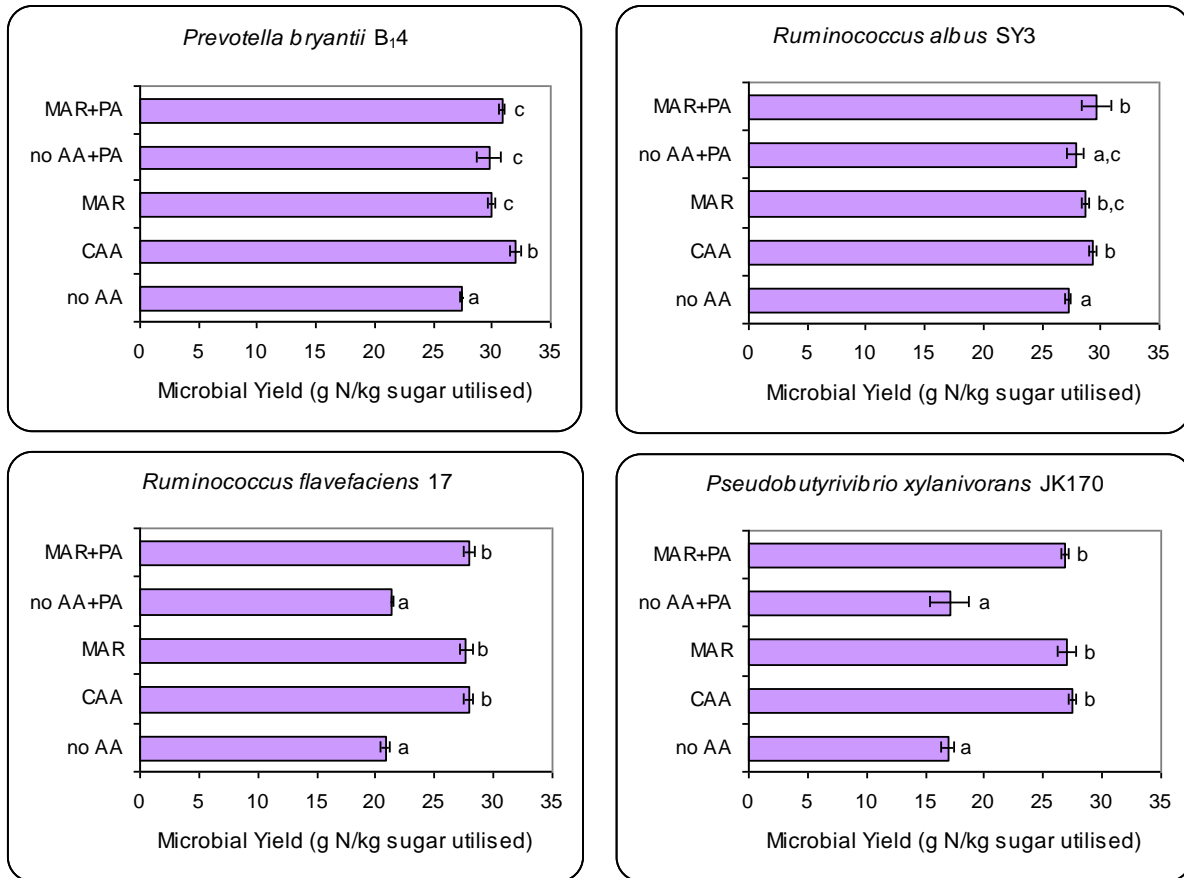
Figure 3. The influence of aromatic amino acids and phenyl acids (phenylacetic and phenylpropionic acids) on microbial yield of rumen bacteria fermenting xylan. Treatments were: no AA=NH$_3$ only, CAA=complete mixture of 20 amino acids, MAR=complete amino acids mixture minus aromatic amino acids, no AA+PA= NH$_3$ only + PA, and MAR+PA=complete amino acids mixture minus aromatic amino acids + PA. The bars represent means calculated from triplicate incubations. Bars with different letters are significantly different ($P<0.05$).

(xylan) utilization reported in these previous studies, and also observed in the present study, can be attributed to species and strain differences.

The addition of complete AA mixtures to a medium with only ammonia as the sole N source seemed to stimulate the growth of bacteria able to utilise xylan (Figure 1). In terms of energetic costs, it is cheaper for the organism to utilise the readily available AA for growth. The observed growth benefit from the AA could also be related to the increased sugar utilization (Table 3). Previous pure culture studies have demonstrated complete AA mixtures being stimulatory to the growth of *R. albus* SY3 and *R. flavefaciens* 17, compared to only ammonia as N source (Atasoglu et al., 2001). In terms of sugar utilization, *P. bryantii* B$_1$4 (a non-cellulolytic species), grown on a medium that contained OSX as energy source, utilised approximately 45% of OSX within 24 h (Miyazaki et al., 1997), which is on the lower side of the sugar utilization range observed in the present study (Table 3). Cotta (1993) observed a 46% utilization of OSX substrate by *R.*

albus 7 after 24 h growth on a trypticase-yeast extract medium. However, Reveneau et al. (2003) noted incomplete degradation of xylan by *R. albus* and suggested that cessation of xylan degradation and growth of *R. albus* may be due to xylose accumulation. This may be the case in the present study, since sugar exhaustion cannot be the cause of cessation of growth as there was a lot of sugar left un-utilized (Table 3). Alternatively, it may be that certain linkages in OSX are either inaccessible due to the insoluble nature of the substrate or the presence of chemical linkages recalcitrant to enzymatic hydrolysis.

The deletion of aromatic AA from a complete AA mixture appeared to decrease the growth of *P. bryantii* B$_1$4, but had less effect on the growth of *P. xylanivorans* JK170 and the two *Ruminococcus* spp. (Figure 1). A similar trend is evident from the sugar utilization analysis (Table 3), suggesting that aromatic AA stimulate the utilization of xylan and growth of *P. bryantii* B$_1$4. Although there are no available records of aromatic AA being

stimulatory to *P. bryantii* B$_1$4, there are previous reports indicating that phenylalanine benefits the growth of *Ruminococcus* spp. (Allison, 1965; Atasoglu et al., 2001). However, these stimulatory benefits were observed while the organisms were fermenting hexose sugar, and may explain the lack of effects on the *Ruminococcus* spp. in the present study where pentose sugar was the only source of energy.

The addition of phenyl acids, to no AA and MAR treatments, seem to have benefited more *P. bryantii* B$_1$4, in terms of growth and xylan utilization, and to a lesser extent *R. albus* SY3 (Figure 1 and Table 3). *Prevotella bryantii* B$_1$4 have been shown to utilise more efficiently xylo-oligosaccharides and xylan fragments (Miyazaki et al., 1997). Since the addition of phenyl acids appears to have remedied the effects of aromatic AA deletion in *P. bryantii* B$_1$4, as shown by increased growth and sugar utilization, it may well be that the bacterium was able to utilise the phenyl acids to synthesize these AA. Several previous studies indicated that phenylalanine or its precursors, PAA and PPA, stimulated growth of *Ruminococcus* spp. (Allison, 1965; Stack et al. 1983; Stack and Cotta, 1986; Morrison et al., 1990). Indeed, one of the widely recognized cellulolytic organisms, *R. albus*, has a requirement for phenyl acids (PAA and PPA) for optimum growth and cellulose degradation (Hungate and Stack, 1982; Morrison et al., 1990; Reveneau et al., 2003). However, when *R. albus* 8 and *R. flavefaciens* FD1 were grown in a medium that contained Trypticase/yeast extract and wheat straw or hydrogen peroxide treated wheat straw, neither bacterial growth nor DM disappearance was affected by addition of PAA and PPA (Odenyo et al., 1991).

A study by Reveneau et al. (2003) on the possible effects of phenyl acids (PAA and PPA) or other components of rumen fluid on xylan degradation and growth of *R. albus* showed that the addition of phenyl acids (or other components present in rumen fluid) had no influence on xylan (OSX and Birchwood) degradation or the growth of any of the *R. albus* strains (8, 7 and B199) tested. In their study they recorded 49% xylan degradation after 24 h incubation of *R. albus* 8 cultures supplemented with phenyl acids. It has been suggested (Allison, 1965 and 1969; Stack et al., 1983) that since phenyl acids, notably PAA, are usually present in the rumen, it may be more economical energetically for the rumen microorganisms to use these acids in the biosynthesis of aromatic AA, notably phenylalanine, than to synthesize the carbon skeleton from carbohydrate or other substances.

Although the strain JK170 has been said to belong to *P. xylanivorans* (Kopecny et al., 2003), it is not a well documented organism. The ability of this organism to grow on xylan and also produce butyrate as a fermentation product, as observed in this study, explains its initial classification as *B. fibrisolvens* (a butyrate producing bacterium). While it appeared to benefit from

the complete AA mixture, neither the deletion of aromatic AA nor the supply of phenyl acids influenced the xylan utilization and growth of this organism (Figure 1 and Table 3).

Volatile fatty acids (VFA) and ammonia

Although the degradation of xylan by numerous ruminal bacteria have been observed, information on the type of fermentation products made is not often reported. In the present study, the main VFA produced during degradation of xylan by all the bacteria is acetate, and butyrate was only produced by *P. xylanivorans* JK170 (Table 1). Most known species of *B. fibrisolvens* produce butyrate (Stewart et al., 1997). Acetate and butyrate have been mentioned as the main fermentation acids of *Butyrivibrio* spp. (Miller and Wolin, 1979).

Pentose sugars can be metabolized by the transketolase and transaldolase reactions of the pentose cycle or by a phosphorolytic cleavage (phospho-ketolase) (Russell, 2002). Since acetate is the main product formed from xylan utilization by the bacterial cultures, it is likely that phospho-ketolase pathway for pentose metabolism might have predominated (Hespell et al., 1987). A study by Hespell et al. (1987) on ruminal bacteria grown in a trypticase/yeast extract medium containing OSX indicated two of the three strains of *B. fibrisolvens* (that is, AcTF2 and D1) produced acetate and butyrate, while the third strain (49) produced butyrate and no acetate. All three strains did not produce propionate, succinate or valerate, but all produced formate. From the same study, *R. flavefaciens* C94 did not also produce propionate, butyrate and valerate, but produced acetate and formate. Their study recorded VFA yields (expressed as moles of acid formed per mole of pentose fermented) that ranged between 0.48 and 1.36.

The increase in acetate production observed in the present study across all the species provided with complete mixtures of AA (Table 1) may be due to increased utilization of xylan as shown by the data in Table 3. The growth benefits derived from the supply of AA by rumen microbes observed earlier could have resulted in increased fermentation/utilization of the energy (xylan) substrate, probably leading to the increased yields of acetate. Moreover, the breakdown of the AA may have also contributed to the increase in acetate and butyrate. The reasons behind the observed decrease in acetate yields, as a result of aromatic AA deletion from a complete mixture, in *P. bryantii* B$_1$4 and *R. albus* SY3 cultures is not clear. However, it is speculated that such a decrease may be due to a reduction in substrate utilization, at least for *P. bryantii* B$_1$4. The supply of phenyl acids seems to induce *P. bryantii* B$_1$4, *R. albus* SY3 and *P. xylanivorans* JK170 to increase acetate production (Table 1). Whether the observed effects are due to the influence of phenyl acids

on microbial growth and enzymic activities or a microbe-substrate interaction or both is not clear.

As expected, the treatments where complete mixtures of AA were supplied recorded highest concentrations of ammonia production (Table 2). Deamination of AA in the rumen by various ruminal bacteria leading to production of significant amounts of ammonia has long been established (El-shazly, 1952; Allison, 1969; Leng and Nolan, 1984; Wallace et al., 1997). Both *Prevotella* and *Butyrivibrio* spp. are involved in the breakdown of AA (other than those directly incorporated during cell growth) to ammonia (Wallace et al., 1997). The high ammonia production observed in *P. bryantii* $B_1$4 (Table 2) may be due to the ability of this organism to break down AA. *Prevotella xylanivorans* JK170, however, appears to be less active. *Ruminococcus* spp. are not generally regarded as AA fermentors and it is unclear as to why there is ammonia production.

The observed reduction in ammonia production when aromatic AA are omitted from a complete mixture (Table 2) could have been due to a reduction in microbial growth rate, at least for *P. bryantii* $B_1$4, leading to a slow down in AA breakdown. Phenyl acids addition caused significant reduction in ammonia production in *P. bryantii* $B_1$4 and *R. albus* SY3 cultures (Table 2), indicating an influence either on the organisms' AA breakdown or uptake of intact AA or maybe both. It may well be that phenyl acids stimulated the organisms to utilise more ammonia, although the mechanism for such stimulation is not clear.

Microbial yield

The microbial yield observed in treatments where AA were supplemented in *P. bryantii* $B_1$4 and *R. albus* SY3 were comparable to the mean yield (30 g microbial N/Kg organic matter digested) reported by ARC (1980). However, yields for *R. flavefaciens* 17 and *P. xylanivorans* JK170 were lower by between 7 and 10%. Microbial yields in treatments that had only ammonia were approximately 9% lower that the ARC (1980) value for *P. bryantii* $B_1$4 and *R. albus* SY3; and 31 and 44% lower for *R. flavefaciens* 17 and *P. xylanivorans* JK170, respectively. The growth yield of all species was stimulated by the supply of complete mixtures of AA (Figure 3), indicating benefit derived from the AA. Previous pure culture studies also showed AA being stimulatory to the growth yield of *R. albus* SY3 and *R. flavefaciens* 17 (Atasoglu et al., 2001). Also, several experiments conducted with mixed ruminal microorganisms confirm the stimulatory effects of AA on microbial growth (Argyle and Baldwin, 1989; Atasoglu et al., 1999; Kajikawa et al., 2002; Atasoglu et al., 2003).

Although the deletion of aromatic AA from a complete mixture did not have significant influence on microbial yield for all the bacteria, there was about 7% decrease in the yield of *P. bryantii* $B_1$4, indicative of the susceptibility

of this organism to omission of these acids. Also, whereas the supply of phenyl acids did not significantly increase yields in all the species, the small benefits observed were greatest for *P. bryantii* $B_1$4 and *R. albus* SY3 (Figure 3), strengthening the earlier observations in growth and sugar utilization results.

The microbial yields (g TCN kg^{-1} sugar utilised) observed in the present study ranged between 17 and 31 (Figure 3). Hespell et al. (1987) reported cell yields (expressed as g of cell protein formed per mole of pentose fermented after 18 h of growth) of 1.1, 5.4, 3.6, 6.0, and 9.5 for *R. flavefaciens* C94 and FD1, *F. succinogenes*, *B. fibrisolvens* H17c and X10C34, respectively, using OSX as energy source.

Other strains of *B. fibrisolvens* that is, 12, A38 and R28 showed higher cell yields of 18.8, 22.7 and 24.0, respectively. Moreover, they also observed that although some organisms (for example, *B. fibrisolvens* HI1c and *R. flavefaciens* C94) appeared to ferment the xylans, cell yields were not as high proportionately (Hespell et al., 1987). On the basis of the final optical densities obtained, Cotta (1993) established that *P. ruminicola* converted xylooligosa-ccharides, prepared from OSX, into microbial cell mass most efficiently, while *B. fibrisolvens* was least efficient.

Conclusions

There was varied response, between species and within strains in the same species, in the growth of the ruminal microorganisms studied, perhaps influenced by ability to utilize xylan. *P. bryantii* $B_1$4, *R. albus* SY3, *R. flavefaciens* 17 and *P. xylanivorans* JK170 grew well, whereas *F. succinogenes* S85, *R. flavefaciens* FD1, and *B. fibrisolvens* JW11 could not grow. The supply of a complete mixture of complete mixtures of AA to the ruminal bacteria, *P. bryantii* $B_1$4, *R. albus* SY3, *R. flavefaciens* 17, and *P. xylanivorans* JK170, stimulated xylan utilization, VFA production and microbial yield.

The deletion of aromatic AA from a complete mixture appears to affect most *P. bryantii* $B_1$4 in terms of growth, yield, VFA production and xylan utilization. The supply of phenyl acids (PAA and PPA) was able to reduce the effects of aromatic amino deletion in the case of *P. bryantii* $B_1$4. However, aromatic AA and phenyl acids do not seem to greatly influence xylan utilization and growth yield of *R. albus* SY3, *R. flavefaciens* 17, *P. xylanivorans* JK170, *N. frontalis* RE1 and *P. communis* P. The results suggest that supplementation of complete mixture of AA is beneficial in stimulating bacterial growth in the rumen.

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Full Length Research Paper

Phenotypic screening of metallo-β-lactamase in multi-drug-resistant *Pseudomonas aeruginosa* using a combined disk diffusion method

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Clinical utilization of carbapenems remains under threat with the emergence of acquired carbapenemase-producing bacteria, particularly metallo-β-lactamases (MBL). *Pseudomonas aeruginosa* which is an important opportunistic pathogen causing nosocomial infections is potentially resistant to different broad-spectrum antibiotics due to its ability to produce metallo-β-lactamase (MBL). In the present 1 year study, 105 isolates of multidrug-resistant (MDR) *P. aeruginosa* were collected from Motahari, Milad and Taleghani hospital laboratories in Tehran. These isolates were screened by the disc diffusion and combined disc methods (imipenem + EDTA; meropenem + EDTA) to determine the capacity of producing MBL. The overall prevalence of MBL-positive isolates was 88.27% using imipenem and imipenem plus ethylene diamine tetra-acetic acid (EDTA) discs, meanwhile 92.73% of 55 randomly selected isolates evaluated phenotypically for the presence of an MBL, using meropenem and meropenem plus EDTA discs as well, were MBL positive. In the light of our results, the rapidly spreading resistance among bacterial populations due to the extensive use of antibiotics is a matter of concern for the optimal treatment of patients and the determination of MBL production of MDR *P. aeruginosa* strains using a simple, reliable and inexpensive testing method is essential in patients suffering from resistant infections.

Keywords: *Pseudomonas aeruginosa*, metallo-β-lactamase (MBL), imipenem, meropenem, ethylene diamine tetra-acetic acid (EDTA), multidrug-resistant (MDR).

INTRODUCTION

Carbapenems are β-lactam group of drugs that are often used as antibiotics of last resort for treating infection due to multiple-resistant Gram-negative bacilli. They are also stable even in response to extended-spectrum and β-lactamases. However, this scenario has changed with the emergence of metallo- β-lactamase (MBL)-producing strains (Jesudason et al., 2005). Resistance to carbape-

nem is now of global concern and being observed more frequently among nonfermenting bacteria, such as *Pseudomonas aeruginosa*.

Multidrug-resistant (MDR) *P. aeruginosa* is responsible for most nosocomial infections in hospitalized patients (Shanthi and Sekar, 2009; Zavascki et al., 2010). These MDR pathogens are capable of producing enzymes that

can inactivate beta-lactams, such as metallo-β- lacta-mase (MBL) that is responsible for a significant propor-tion of carbapenem resistance in these bacteria (Borgianni et al., 2010; Moya et al., 2009). These enzymes can hydrolyse all classes of β-lactam drugs and withstand neutralization by β-lactamase inhibitors (Wan Nor Amilah et al., 2012). MBLs are β-lactamase enzymes that possess metal ion(s) at their active sites.

These enzymes require zinc for their catalytic activity and are inhibited by metal chelators, such as ethylene diamine tetra-acetic acid (EDTA) (Livermore and Woodford, 2000). The genes responsible for the produc-tion of MBLs are typically part of an integron structure and are carried on transferable plasmids or can also be part of the chromosome (Wan Nor Amilah et al., 2012).

MBL-producing Gram-negative bacilli have been increas-ingly reported in Asia, Europe, Latin American and the United States (Chu et al., 2001; Iyobe et al., 2000; Kurokawa et al., 1999; Miriagou et al., 2003; Toleman et al., 2004). The proportion of carbapenem resistance attri-buted to MBLs has increased significantly as well; the presence of MBLs accounted for resistance in 43.9% of Brazilian and 39.1% of Italian imipenem-resistant *P. aeruginosa* isolates (Toleman et al., 2005). These per-centages represent a dramatic escalation in the fraction of resistance caused by these enzymes.

With the increase in worldwide occurrence, types, and rate of dissemination, early detection of MBL isolates is critical. The benefits of early detection include timely imple-mentation of strict infection control practices as well as clinical guidance regarding the potential risks for thera-peutic failure. Although polymerase chain reaction (PCR) is highly accurate and reliable, its accessibility is often limited to reference laboratories (Franklin et al., 2006). Several non-molecular techniques have been studied, mostly taking advantage of the enzyme's zinc depen-dence by using chelating agents, such as EDTA to inhibit its activity (Bush and Fisher, 2011; Bush and Jacoby, 2010; Deshmukh et al., 2011).

Since the rapid spread of bacterial resistance due to the extensive use of antibiotics remains a matter of concern for the optimal treatment of patients, the evalua-tion and use of a simple, reliable and inexpensive testing method for screening of MBL-producers in routine labora-tory has become necessary.

In this study, our aim was to determine the incidence of metallo-β-lactamase (MBL) enzymes in the *P. aeruginosa* clinical isolates using a combined disk diffusion method.

MATERIALS AND METHODS

Bacterial isolates collection and characterization

In the present study, 105 *P. aeruginosa* clinical isolates were collected over a period of 1 year (January 2012 to January 2013) from Motahari, Milad and Taleghani hospital laboratories in Tehran. The samples were immediately transported in transport culture media under standard conditions to the central laboratory of the

Infection Research Center. *P. aeruginosa* ATTCC27853 was used as the negative control. Determination of *P .aeruginosa* strains was confirmed by standard biochemical tests (Wan Nor Amilah et al., 2012).

Antimicrobial susceptibility test

Antimicrobial susceptibility testing was performed on Mueller-Hinton agar plates with: Piperacillin (100 μg), Ticarcillin (75 μg), Carbenicillin (100 μg), P-Tazobactam (110 μg), Ceftazidime (30 μg), Aztreonam (30 μg),Imipenem (10 μg), Meropenem (10 μg), Colistin sulphate (10 μg), Gentamicin (120 μg),Tobramycin (10 μg), Amikacin (30 μg), Ciprofloxacin (5 μg) , Levofloxacin (5 μg) and Norfloxacin (10 μg) (Thermo Scientific™, USA) discs by disc diffusion method and interpreted as per Clinical and Laboratory Standards Institute (CLSI, 2012) recommendations. Despite the different definitions of MDR micro-organisms (Falagas et al., 2006), MDR was used in this study for isolates that were resistant to at least three classes of antibiotics.

Phenotypic detection of MBL

Screening of MBL-producing isolates was performed using a combined disk diffusion method. The isolates were evaluated phenotypically for the presence of a metallo-β-lactamase (MBL), using the metal chelating agent: EDTA (Vahdani et al., 2012). Identification of MBL activity was performed by carbapenem-EDTA combined disk method. Two imipenem or meropenem [IMP (10 μg), MEM (10 μg)] discs were applied to a Muller Hinton agar plate inoculated with a bacterial suspension of 0.5 McFarland turbidity standards, and 10 μl of a sterile 0.5 M EDTA (pH 8.0) solution was applied to one disk. A sterile 6-mm filter paper disc to which 10 μl of 0.5 M EDTA was applied was used to determine if EDTA alone might inhibit the growth of the test isolates. The plates were incubated at 35°C under ambient air for 18 h.

The zones of inhibition around the IMP and IMP-EDTA discs were measured for all 105 isolates, however the zones of inhibition around the MEM and MEM-EDTA discs were measured for 55 isolates selected randomly and zone increases of ≥7 mm in the presence of EDTA were noted and interpreted as indicative of an MBL phenotype on the basis of criteria described previously (Bashir et al., 2011; John and Balagurunathan, 2011; Mochon et al., 2011).

RESULTS

In this study, 105 clinical isolates of *P. aeruginosa* were collected from three hospital laboratories in Tehran during 1 year.

All samples were MDR. Antimicrobial susceptibilities were determined according to the interpretative criteria of the CLSI guidelines (Table 1). High resistance to all antimicrobial drugs was observed except for Colistin (3.81%) and Gentamicin (67.62%).

The overall prevalence of MBL-positive isolates was 88.27% using IMP and IMP-EDTA discs, meanwhile 92.73% of 55 randomly selected isolates evaluated pheno-typically for the presence of a metallo-β-lactamase (MBL), using MEM and MEM-EDTA discs as well, were MBL positive.

Among 105 isolates, seven isolates showed different MBL producing pattern using IMP and IMP-EDTA or MEM

Table 1. Antibiotic resistance among multi- drug- resistant *Pseudomonas aeruginosa* isolated from 3 hospital laboratories in Tehran.

Antibiotic Group	Antibiotic	Sensitive number (%)	Intermediate number (%)	Resistant number (%)
Penicillins	Piperacillin	2(1.90%)	7(6.67%)	96(91.43%)
	Ticarcillin	-	1(0.95%)	104(99.05%)
	Carbenicillin	-	1(0.95%)	104(99.05%)
β-lactam/β-lactamse inhibitor combinations	P-Tazobactam	1(0.95%)	15(14.28%)	89(84.76%)
Cephems	Ceftazidime	11(10.48%)	3(2.86%)	91(86.67%)
Monobactams	Aztreonam	1(0.95%)	7(6.67%)	97(92.38%)
Carbapenems	Imipenem	-	-	105(100%)
	Meropenem	-	-	105(100%)
Lipopeptides	Colistin	101(96.19%)	-	4(3.81%)
Aminoglycosides	Gentamicin	10(9.52%)	24(22.86%)	71(67.62%)
	Tobramycin	3(2.86%)	1(0.95%)	101(96.19%)
	Amikacin	4(3.81%)	2(1.90)	99(94.3%)
Fluoroquinolones	Ciprofloxacin	1(0.95%)	2(1.90%)	102(97.4%)
	Levofloxacin	2(1.90%)	1(0.95%)	102(97.4%)
	Norfloxacin	1(0.95%)	3(2.86%)	101(96.19%)

Table 2. Different MBL producing pattern using IMP and IMP-EDTA or MEM and MEM-EDTA discs among 7 *Pseudomonas aeruginosa* isolates.

Sample number	IMP+IMP-EDTA	MER+MER-EDTA
2	-	+
5	-	+
22	-	+
23	+	-
24	-	+
35	+	-
37	-	+

+, MBL positive; -, MBL negative

and MEM-EDTA discs (Table 2).

DISCUSSION

Carbapenems were the effective antibiotics for MDR gram-negative bacteria infections, especially in high-risk hospital settings (Qing et al., 2012). However, resistance is the result of different mechanisms, such as MBL production by micro-organisms. These enzymes belong to Ambler class B β-lactamases based on their amino acid sequence homology and to group 3 according to the Bush classification based on their substrate profiles (imipenem hydrolysis) (Ambler, 1980; Bush et al., 1995). The genes responsible for MBL production may be chromosomal or plasmid mediated (Wan Nor Amilah et al., 2012). MBL enzymes are inhibited by EDTA

(Livermore and Woodford, 2000). The rapid detection of MBL positive isolates is necessary to control infection and to prevent their dissemination.

The increasing use of extended spectrum antibiotics such as carbapenems would provide the selective pressure for selection of these enzymes (Irfan et al., 2008). In many studies across the world, different resistance ranges (4-60%) have been reported towards carbapenems. For instance, the incidence of MBL production in *P. aeruginosa* has been reported to be 10-30% from a variety of clinical specimens across India (Deshpande et al., 2010). However, in a study by Lagatolla et al. (2004) in Italy, 70% MBL was found in *P. aeruginosa*. Our prevalence of MBL in *P. aeruginosa* does not correlate with other studies across the country. In a study by Vahdani et al. (2012), 38 % of MBL production was reported in *P. aeruginosa*, and in another

study performed in Ahwaz, 19.51% of *P. aeruginosa* strains isolated from burn patients were reported as MBL producers (Khosravi and Mihani, 2008), whereas in the study of Manoharan et al. (2010), in accordance with our results, 87.8% of *P. aeruginosa* isolates were MBL-positive, using a combined disk diffusion method. This variation reflects the different diagnostic methods and the different rates of antibiotics used in different hospitals.

Although CLSI has recommended IMP- EDTA for MBL investigation in *P. aeruginosa* (CLSI, 2012), as mentioned earlier, in our study, two *P. aeruginosa* isolates were MBL - positive using IMP and IMP- EDTA discs while these isolates were negative for MBL using MEM and MEM -EDTA discs; this contrast was observed for five other isolates, conversely. Thus, using different subclasses of carbapenems such as imipenem + EDTA and meropenem + EDTA together for investigating MBL-positive isolates by combined disk diffusion method, seems to be necessary.

The emergence of MBL-mediated resistance in our country, Iran, is a matter of concern for the treatment of patients (Vahdani et al., 2012). The phenotypic screening of resistance is an important step for epidemiological purposes and for developing policies for effective infection control measures in order to manage and prevent the spread of resistant strains. We suggest accurate surveillance, especially of MBL, in MDR *P. aeruginosa* isolates as an important step for optimal antibacterial treatment in the future.

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Full Length Research Paper

São Paulo Zoo composting as a source of bacteria with bioremediation potential

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As the world population increases, the need for energy resources increases and for decades, petroleum has sustained this demand. Oil spill during petroleum extraction and processing has a negative impact on the environment. Methods that can decrease the impact of xenobiotic compounds on the environment have been developed. Among many bioremediation methods described, microbial remediation is of great importance. Our previous work demonstrated that Organic Waste Composting Unit (OWCU) located at the São Paulo Zoo Foundation (FPZSP) had a great diversity of microorganism. In the present work, we viewed this environment searching for xenobiotic degrading microorganisms by sampling the composting at various stages of the process with the aim of isolating the bacteria that would break down n-hexadecane, a model compound for hydrocarbon degradation. Two bacterial collections were assembled and tested in a 96-well plate model using n-hexadecane as a sole carbon source. Among the 418 isolates screened, eight were selected based on their ability to assimilate n-hexadecane. Molecular identification revealed their genus and species which are associated with xenobiotic degradation activities in different microbial consortia. However, these microorganisms have not being isolated from the same transforming process. Future studies with these isolates may shed light on the bacteria hydrocarbon degradation mechanism.

Key words: Composting, crude oil degrading microorganism, bioremediation, n-hexadecane degrading bacteria.

INTRODUCTION

The increase in population and consumption has led to large amounts of organic waste accumulation, which has been a serious problem to manage in both urban and rural areas. Composting is an alternative technique for disposing organic waste and avoiding its accumulation. The end product generated is a natural fertilizer, which can be used in agriculture closing a self-sustainable cycle. The São Paulo Zoo (São Paulo, Brazil) is a good example where its Organic Composting Production Unit (OWCU) transforms about 2000-2500 kg/day of organic material into fertilizer within a ninety-day period.

Composting is an ancient biological process carried out by a microorganism consortium. The microbial metabo-lism changes the substrate composition over time, which in turn reflects on microbial population structure. In this process, the interaction between biotic and non-biotic factors leads to constant transformation of the complex microbial community over time, which is the mechanism underlining organic matter transformation (Schulze, 1962; Waker et al., 1999; Dees and Ghiose, 2001; Hua et al., 2010). Therefore, composting is a rich source for microbial diversity studies suitable for investigation with the aim of getting a generation of biotechnological pro-ducts such as microorganisms that are capable of degrading xenobiotic contaminants in soil.

In the past years, the world has witnessed many oil spill

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disasters that contaminated water and soil. Among several means for soil and water decontamination, bioremediation has gained a lot of attention because living organisms convert contaminant into chemical compounds more friendly to the environment (Rojo, 2009; Zhang et al., 2010; Nagata et al., 2010).

Crude oil is a complex mixture of hydrocarbons and other organic compounds (Zhang et al., 2010). Saturated hydrocarbon alkanes are the major fraction constitutes of crude oil and depending on their oil source, they can reach up to 50% of its constitution. These molecules are chemically inert and can be used by microorganisms as carbon source. Therefore, researchers have used alkanes as single carbon source to isolate microorganisms with bioremediation potential (Rojo, 2009).

Our previous work (Bitencourt et al., 2010; Pascon et al., 2011; Farage-Martins et al., 2013) has shown the potential of the OWCU compost as a source of interest and useful microorganisms. Therefore, the aim of the present work was to explore the microbial diversity present in the zoo composting mixture in order to select and characterize those that can use the model compound n-hexadecane (n-alkane) as sole carbon source. We expected that these microbes would have a potential for bioremediation and will be used in further degradation studies.

MATERIALS AND METHODS

Microorganism collection

Two microorganism collections were screened in this work. CL001 collection was generated previously as described by Bitencourt et al. (2010) and Pascon et al. (2011), whereas CL002 collection was generated for this work and comprised samples taken at a defined time points from a single compost cell. Each composting cell unit has eight cubic meters and it is assembled with organic waste collected from the São Paulo Zoo (Bitencourt et al., 2010), which is converted into fertilizer after approximately 90 days. During this process, the temperature can rise up to seventy degrees Celsius and water sprinkling is used to bring the temperature down. When the temperature drops below fifty-five degrees Celsius, the cell is revolved to recover its aerobic conditions and humidity in the range of 40-60%.

The whole process is finalized when the cell temperature becomes stabilized at about fifty degree Celsius for several days. The time points chosen aimed to cover the three stages of composting described are as follows: 1) soon after the composting cell unit was assembled at time zero (T_0), 2) 82 days after T_0 during the compost mixing and 3) 99 days after T_0 when the process was finished and the temperature was below fifty degrees Celsius. The sampling at each time point was performed as described by Bitencourt et al. (2010).

Microorganism isolation

Two isolation protocols were employed in this work. The first one was described by Bitencourt et al. (2010) and was isolated in rich medium without selecting xenobiotic degradation bacteria. This isolation method was used to assemble collection CL001. The second microbial collection (CL002) comprised bacterial isolates that were obtained by direct isolation as in CL001 and microorganisms capable of growing in n-hexadecane enriched medium as described in the study of Mrozik and Piotrowska-Seget (2010) and Vasconcellos et al. (2010). In brief, five grams of compost was added to 10 mL of saline (0.9% NaCl); it was mixed and after 1 h at room temperature, an aliquot of 5 mL was used to inoculate 500 mL of minimal medium M9 (41) containing 1% (w/v) of glucose and 0.25% (v/v) of n-hexadecane (Sigma, cat.# H6703). The culture was incubated at 30°C for 72 h with 150 rpm. The cells were collected by centrifugation at 3220 g for 2 min (Eppendorf 5810R with *swing* rotor A-4-62) and added to a fresh 500 mL of minimal medium M9 supplemented with 0.5% (v/v) of n-hexadecane. The culture was incubated for 48 h at 30°C with 150 rpm.

Again, the cells were recovered as described above and brought to suspension and inoculated in 500 mL of minimal medium M9 supplemented with 1% (v/v) of n-hexadecane. The culture was incubated for 48 h at 30°C with 150 rpm. The growth of the cultures was checked daily in a spectrophotometer (Bel photononic UV2000) at 630 nanometers. Isolation of microorganisms was performed as described in the study of Bitencourt et al. (2010) in nutrient agar for each step of the enrichment procedure at three time points: at time point zero (T_0) when the culture was set up, at time point one (T_1) after 24 h of growth and at time point two (T_2) after 48 h of growth. A T_3 time point was taken only for the first step of enrichment since this culture has grown for 72 h. All the experiments were made in triplicate.

n-Hexadecane as sole carbon source

From a freshly grown bacterium culture, a small inoculum was removed and added to 5 mL of LB broth. This culture was grown for 12-16 h at 30°C with 150 rpm. Then a 1:10 culture dilution was made and its optical density was determined in a spectro-photometer at 630 nm (Bel photononic UV2000). The optical density was adjusted to 0.26. An aliquot of 15 µL was added to wells containing 135 µL of minimum medium M9 with 1% of n-hexadecane in triplicate. The culture was incubated for 48 h at 30°C with 150 rpm. Then the cell density was analyzed in a spec-trophotometer (Bel photononic UV2000) at 630 nanometers of optical density. The cell activity was determined by adding 20 µL of a 1 mg/mL solution of Thiazolyl Blue Tetrazolium Bromide MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Merck® cat. # 1117140001] and incubating at room temperature with 150 rpm for one hour. The wells that yielded purple color were scored as positive for cell growth, whereas no cell activity was indicated by a yellow color (French et al., 1998).

Bacteria genomic DNA isolation

Bacterial genomic DNA isolation was performed using the QIAamp DNA Mini Kit (Qiagen cat. #51304). The DNA integrity was determined in a 0.8% agarose gel in a 1X TAE (Tris, Acetate and EDTA) buffer as described by Sambrook et al. (1989).

Amplification of the ribosomal small subunit (16S) by PCR

The ribosomal small subunit (16S) was amplified by PCR using the pair of primers forward (5' GTGCCAGCMGCCGCGG 3') and was reversed (5' ACGGGCGGTGTGTRC 3'); the reaction conditions were described by Lane (1991) and Borneman and Hartin (2000). The PCR product yielded was checked in 0.8% agarose gel in a 1X TAE (Tris, Acetate and EDTA) buffer as described by Sambrook et al. (1989). Prior to BigDye sequencing (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems), the PCR product was purified by QIAquick PCR Purification Kit (Qiagen, cat. #28106).
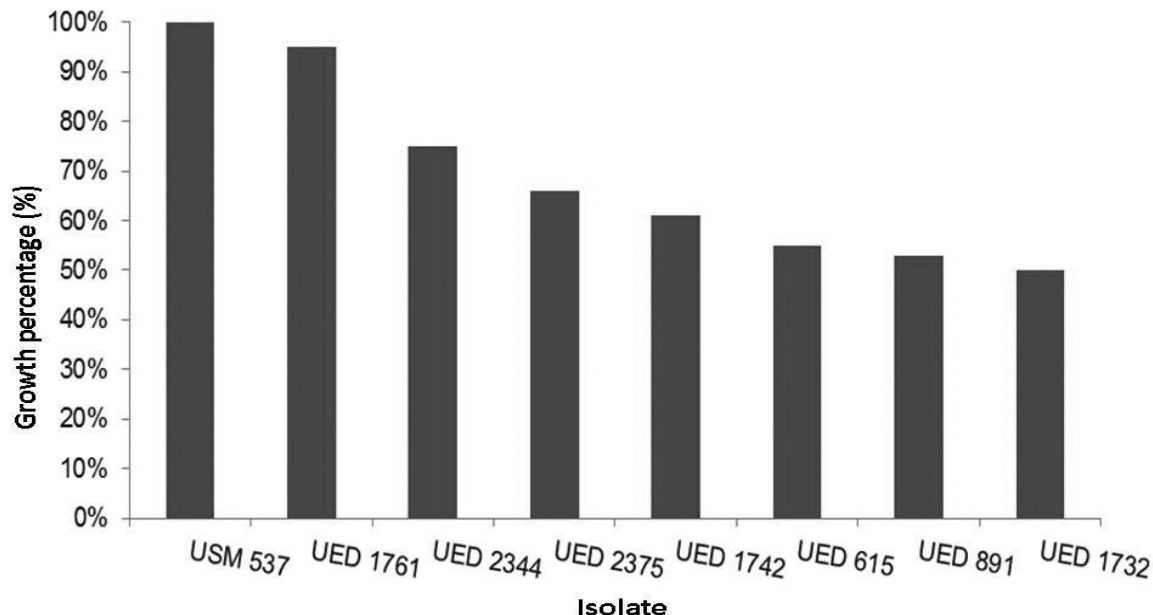
Figure 1. Growth rate of eight isolates in minimal medium M9 supplemented with n-hexadecane on a 96 well plate assay. Isolate USM537 had the best growth rate under this condition when when compared with others.

The sequences yielded by the BigDye sequencing were blasted against the following databases: NCBI (National Center for Biotechnology Information) and the Ribosomal Database Project - Release 10 (http://rdp.cme.msu.edu/).

RESULTS AND DISCUSSION

Micro-organisms collections and the 96 well plate assay for n-hexadecane degradation

Two collections (CL001 and CL002) of isolated microorganisms from composting were subjected to n-hexadecane assimilation as carbon source. CL001 yielded 259 bacteria as described by Pascon et al. (2011). On the other hand, CL002 collection was obtained by sampling a single compost cell unit at three different time points that covered three important stages during the composting process as follows: at time point zero (T_0) where the temperature was 60.4°C with pH 6.0; at time point one (T_1) it was sampled 64 days after the initiation of the process (T_0) and soon after a compost cell unit mechanical aeration where temperature was 56.6°C with pH 7.0. The time point two (T_2) was taken 99 days after T_0 where the temperature was about 49.6°C with pH 6.5. Even though the composting material presented high temperature (60.4 to 49.6°C) at different collection points, the bacteria isolation for CL002 was performed at 30°C as previously described in our laboratory (Pascon et al., 2011). And also, this temperature was employed because our aim was to isolate microorganisms capable of degrading substances that contaminate the environment, therefore thermopiles were not the subject for this

purpose. The CL002 collection comprised 159 bacteria, which were obtained by direct isolation as described for CL001 (Pascon et al., 2011) and by the enrichment methodology described previously by Mrozik and Piotrowska-Seget (2010) and Vasconcellos et al. (2010). Therefore, a total of 418 isolates were tested for their ability to grow on n-hexadecane, an n-alkane, as a sole carbon source. Among all the bacteria tested, 79 (18.9%) were able to degrade n-hexadecane to obtain energy. The cell growth was confirmed by the addition of MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide]. The USM537 isolate had the best growth pattern under these conditions and therefore was considered as the reference strain for comparing the others. Figure 1 shows seven isolates that were able to grow at least 50% of the USM537 on n-hexadecane containing minimal medium M9 on a 96 well plate assay. Among these eight isolates (including USM537), two are from CL001 collection and six are from CL002 (Table 1).

Molecular identification

In order to uncover the genus and/or species of these bacteria, molecular identification was performed. A PCR fragment comprising the ribosomal small subunit (16S) DNA was amplified and sequenced. These sequences were used to conduct similarity searches at the GeneBank (National Center for Biotechnology Information) and Ribosomal Database Project - Release 10 (http://rdp.cme.msu.edu/) using BLAST. The results are presented in Table 1.

As seen in Table 1, we identified eight isolates as good

Table 1. Molecular identification of eight isolates that are able to grow on minimum medium M9 supplemented with n-hexadecane as sole carbon source.

Isolate	16S	Collection	Max. ID (%)
USM 537	*Klebsiella oxytoca*	CL001	99
UED 615	*Enterobacter cloacae*	CL001	99
UED 891	*Klebsiella sp.*	CL002	100
UED 1732	*Sphingobacteria sp.*	CL002	96
UED 1742	*Isoptericola sp.*	CL002	99
UED 1761	*Isoptericola variabilis*	CL002	99
UED 2344	*Microbacterium sp.*	CL002	99
UED 2375	*Bacillus licheniformis*	CL002	99

candidates for bioremediation of crude oil and its derivatives from contaminated locations employing n-hexadecane (n-alkane) as an indicator. According to many publications, these genera and species have been reported to be involved in many bioremediation processes.

The genus, *Klebsiella* has ubiquitous distribution in terms of its habitat like sewage, drinking water, soils, surface waters, industrial effluents and vegetation. Also, this bacterium has been described as resident or transient in the gastrointestinal tract. Thus, *Klebsiella* is also known as common opportunistic pathogen for humans and other animals. Six genera of *Klebsiella* are associated with several environments as follows: *Klebsiella pneumonia* found in humans, animals, sewage and polluted waters and soils; *Klebsiella oxytoca* is often associated with most habitats; *Klebsiella terrigena* found in unpolluted soils and surface waters, drinking water and vegetation; *Klebsiella planticola* is associated with sewage, polluted surface waters, soils and vegetation; and *Klebsiella ozaenae/ Klebsiella rhinoscleromatis* is rarely detected; however is primarily associated with humans (Bagley, 1985).

Moreover, the genus, *Klebesiella* is known to be involved in bioremediation of several pollutants. Li and Gu (2007) have isolated *Kleibesiella oxytoca* from mangrove and they showed that this strain in consortium with *Methylobacterium mesophilicum* can rapidly metabolize dimethyl isophthalate (DMI) as sole carbon source. DMI is used in the sodium dimethyl isophthalate-5-sulfonate (SIPM) production, which is used to enhance the chromaticity of polyethylene terephthalate (PET). Khalid et al. (2009) have isolated *K. oxytoca* and two other microorganisms (*Acinetobacter* sp. and *Citrobacter freundii*) that were able to grow on 4-nitroaniline from textile dye wastewater. This microorganism consortium was able to remove 100% of 4-nitroaniline in 72 h. Kim et al. (2009) have isolated *K. oxytoca* from phenanthrene (PHE) which is a polycyclic aromatic hydrocarbon composed of three fused benzene rings. Together with *K. oxytoca*, they also isolated *Acinetobacter baumannii* and *Stenotrophomonas maltophilia*. Used alone, *K. oxytona*

was able to remove only 11% of the phenantrhere contaminant, whereas in a consortium, the three microorganisms degraded 80% PHE in 360 h. It is interesting to note that *K. oxytoca* is reported by Khalid et al. (2009) and Kim et al. (2009) as having the ability to degrade compounds like PHE and 4-nitroaniline which have cyclic closed carbon rings. In the present report, this microorganism was isolated in an enrichment process with a linear hydrocarbon molecule. However, other researchers have reported the isolation and ability of this microorganism to grow and degrade crude oils, which are rich in alkanes (Zhang et al., 2010). Rajasekar et al. (2010) reported the isolation of *K. oxytoca* in diesel and naphtha pipelines in the Northwest and Southwest regions in India. They have also isolated other 10 bacteria (*Serratia marcescens* ACE2, *Bacillus subtilis* AR12, *Bacillus cereus* ACE4, *Pseudomonas aeruginosa* AI1, *Pseudomonas stutzeri* AP2, *Bacillus litoralis* AN1, *Bacillus* sp., *Bacillus pumilus* AR2, *Bacillus carboniphilus* AR3 and *Bacillus megaterium* AR4). Chamkha et al. (2011) have isolated *K. oxytoca* from Tunisian offshore oil field. Their isolate was capable of degrading a wide range of aliphatic hydrocarbons from C13 to C30. In 45 days, *K. oxytoca* was able to degrade 75% of n-alkanes; however with the addition of a surfactant (Tween 80), the degradation rate reached 98%. Also, they showed that this isolate was able to completely metabolize aromatic compounds within 24 h. Interestingly, our isolate failed to grow on phenol containing medium as sole carbon source (data not shown).

The species, *Enterobacter cloacae* has the ability to degrade various xenobiotic compounds. It has been reported that it can also accumulate heavy metal (Fang et al., 2010; Kanaly et al., 2000; Turgay et al., 2012; Rajasekar et al., 2010), grow on explosives such as pentaerythritol tetranitrate (PETN) and 2,4,6-trinitrotoluene (TNT) as sole nitrogen source (Binks et al., 1996; French et al., 1998) and it can decontaminate water from herbicides (Chena et al., 2009; Sakultantimetha et al., 2001; Ngigi et al., 2012). Also, this microorganism has been isolated from materials contaminated with crude oil. Saadoun (2002) isolated six

different species of bacteria growing on soil contaminated with crude oil and among them he reported that *E. cloacae* was able to degrade diesel as well. Zhang et al. (2009) reported that a consortium between *E. cloacae* and *Cunninghamella echinulata* elevated the biodegradation of total petroleum hydrocarbon (TPH) from 29.2 to 48.0% after about 20 days. They added wheat straw to the contaminated soil and after 45 days, the biodegradation ratio of TPH reached 75%. Wu et al. (2011) reported that applying wheat straw and a different consortium (*E. cloace*, *Pseudomonas* sp. and *Rhodothermus* sp.) showed an increase in the overall degradation ratio from 44 to 56% after 56 days of treatment.

Sphingobacterium is known by its ability to degrade pollutants such as pesticides, textile dyes and petroleum and its derivatives (Kanaly et al., 2000; Carvalho et al., 2002; Macbeth et al., 2004; Fang et al., 2010; Nagata et al., 2010; Tamboli et al., 2010; Janbandhu and Fuleka, 2011). Polycyclic aromatic hydrocarbons (PHA) can be found in oil, coal and tar deposits, and they are also produced as byproducts of fuel burning. These compounds are found in contaminated soils and sediments and as pollutants; the concerns fall on the fact that they can be carcinogenic, mutagenic and teratogenic (Wong et al., 2004).

Bacteria from the genus, *Sphingobaterium* can degrade PHA. Janbandhu and Fulekar (2011), using an enrichment protocol, have identified *Sphingobacterium* sp., *Bacillus cereus* and a novel bacterium, *Achromobacter insolitus* (MHF ENV) within a consortium which was able to degrade phenanthrene from a three decade old petrochemical refinery. In their experimental conditions, the consortium was able to mineralize 100% of phenanthrene at 100 mg/L in 14 days.

We have also identified *Isoptericola variabilis* (formely know as *Cellulosimicrobium variabile* (Stackebrandt et al., 2004) and the isolate UED1742 (*Isoptericola* sp.) as microorganisms that can use n-hexadecane as a sole carbon source. Radwan et al. (2010) studied crude oil-coated gravel particles covered with blue-green biofilms in the Arabian Gulf Coast in autumn, winter and spring. They have isolated and characterized many bacteria that were able to grow on crude oil supplemented or not with a nitrogen source. Among the many microorganisms isolated they found *Isoptericola* sp. that was able to degrade hydrocarbon as sole carbon source in the presence and absence of nitrogen with the following hydrocarbon attenuation percentages: 22.4±0.1 and 27.1±2.0%, respectively.

Microbacterium species have been isolated as part of a microbial consortium from soil contaminated with xenobiotics such as dyes, heavy metals, pesticides and crude oil and its derivatives (Lal et al., 2010; Waranusantigul et al., 2011; Chanthamalee and Luepromchai, 2012; Juárez-Ramirez et al., 2012; Turgay et al., 2012; Shin et al., 2012; Simarro et al., 2013). Lal et al. (2010) have isolated a micro-bacterium from a microorganism con-

sortium growing on hexachlorocyclohexane-contaminated soil.

Waranusantigul et al. (2011) and Turgay et al. (2012) have isolated from plant rhizosphere, *Microbacterium* species that were able to promote heavy metal accumulation and plant growth. They concluded that the rhizosphere bacteria have the potential to improve the efficiency of phytoremediation of plumb and nickel-contaminated sites. Shin et al. (2012) have isolated thirty-seven carbofuran-degrading bacteria through enrichment processes from a variety of rice paddy soils and among them they found a species of *Microbacterium* (*M. oxydans*) that was able to degrade this pesticide. *Microbacterium* was isolated from a biofilm capable of degrading 4-aminonaphthalene-1-sulfonic acid used in the process of decolorization of azo dyes (Juárez-Ramirez et al., 2012). Also, *Microbacterium* species were isolated from different environments and were shown to be able to degrade polycyclic aromatic hydrocarbons (PAHs), crude oil, hydrocarbon used as boat lubricant and as single carbon source (Chanthamalee and Luepromchai, 2012; Hassanshahian et al., 2012; Simarro et al., 2013).

Among the microorganisms that are able to grow on n-hexadecane as sole carbon source, we have identified *Bacillus licheniformis*. This *Bacillus* species is known to participate on organic matter conversion (composting) as reported by Haruta et al. (2002). This microorganism can produce bioemulsifier that helps to enhance crude oil recovery (Suthar et al., 2008), biodegrade textile dyes (Lu et al., 2012) and crude oil and its derivatives (da Cunha et al., 2006; Itah et al., 2009). However, its ability to degrade crude oil and its derivatives (such as jet fuel) is shown when *Bacillus licheniformis* grows within a microbial consortium with other microorganisms.

The present work used enrichment protocol to increase the amount and/or diversity of microorganisms and to obtain results as those achieved and described by many authors. It was found that the genus diversity for n-hexadecane degrading microorganisms CL002 was higher as compared to CL001 (data not shown).

The degradation of all the organic matter present in the zoo composting cells is not done by a single microorganism but by the synergistic action of several of them. Therefore, our findings are in agreement with several publications that report that microorganism consortium is capable of transforming the environment where they are placed and/or found (Binks et al., 1996; Kanaly et al., 2000; Carvalho et al., 2002; Haruta et al., 2002; Li and Gu, 2007; Itah et al., 2009; Khalid et al., 2009; Kim et al., 2009; Fang et al., 2010; Hua et al., 2010; Radwan et al., 2010; Rajasekar et al., 2010; Jandandhu and Fulekar, 2011; Sakultantimetha et al., 2011; Waranusantigul et al., 2011; Chanthamalee and Luepromchai, 2012; Juárez-Ramirez et al., 2012; Turgay et al., 2012; Shin et al., 2012; Simarro et al., 2013).

The results indicate that the microbial consortium has a promising application in bioremediation of xenobiotic con-

taminated environments. Thus, the eight isolates that are object of this work should in a future study be tested altogether in a more challenging environment for degradation of crude oil and its derivatives.

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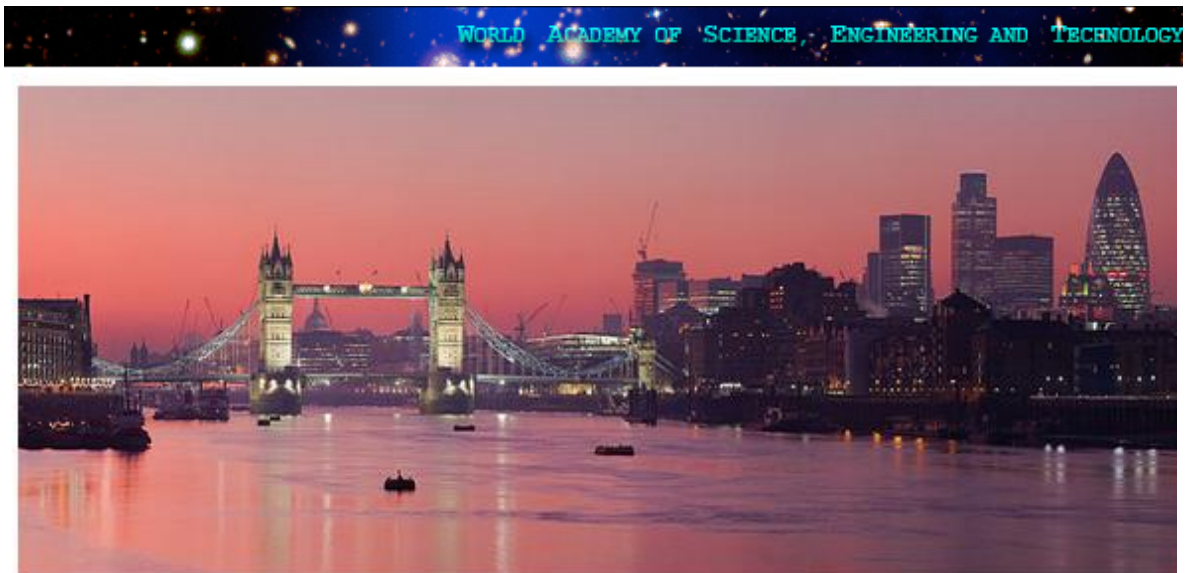
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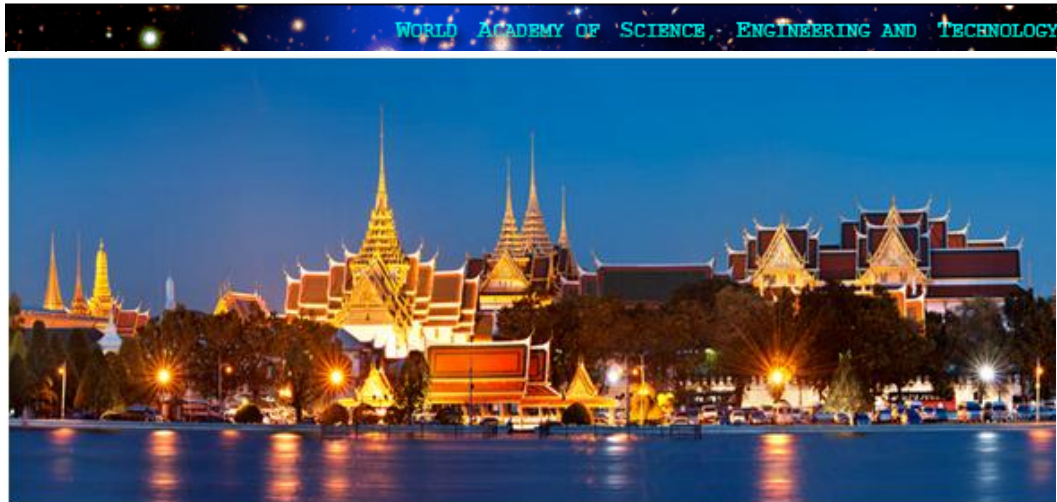
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