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Isolation and Molecular Characterization of Xylanase-Producing Bacteria from Drain Water

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Abstract: The xylanase-producing bacterial strains were isolated from drain water. The strains were isolated on xylan-agar media and screening was carried out by β-xylanolysis method. The strains were further confirmed by DNS-method. The isolated strains were identified as *Aeromonas* on the basis of catalase, oxidase, gram staining and morphological cultural characteristics as well as by different biochemical tests. Multi-drug resistant of these strains were confirmed by antibiotic spread plate method. Disc diffusion method and antibiotic spread plate method showed the same multi-drug resistant pattern. Plasmid DNA was isolated from maximum xylanase-producing strain. Agarose gel electrophoresis of the isolated plasmid DNA in 0.8% agarose showed the presence of only one 30 kb plasmid. The plasmid DNA was transformed into nonxylanase-producing sensitive *E. coli* LE392. The transformants showed xylanase activity as well as multi-drug resistance. Xylanase activity and multi-drug resistance in *Aeromonas* strain was proposed to be plasmid mediated.

Key words: *Aeromonas* sp., plasmid, multidrug resistant, xylanase

Introduction

β-1, 4-xylan, the major component of hemicellulose, are heterogeneous polysaccharides consisting of a homopolymeric backbone of 1,4-linked β- D-xylopyranose unit and short chains including O-acetyl, α-L-arabinofuranosyl and α-D-glucuronyl residues (Whistler and Richards, 1970). It has been shown that bacteria and fungi use β-1, 4-xylan as a carbon source using xylanolytic enzymes such as β -1, 4-xylanases, β-xylosidases and β-glucuronidases (Sunna and Antranikian, 1997). β-1, 4-xylanases (E.C.3.2.1.8) are the key enzymes that hydrolyze the backbone structure of β-1, 4-xylans. A number of β-1, 4-xylanases have been purified from a wide range of microorganisms including *Bacillus* spp., *Clostridium* spp. *Streptomyces* spp., *Aspergillus* spp. and *Trichoderama* spp. (Wong *et al.*, 1988). All these microorganisms produce multiple xylanases, implying a strategy for effective hydrolysis of β-1, 4-xylan. Each enzyme may have a specialized function in the degradation of the complex polysaccharides and specialized functions of individual xylanases may be useful for applications of food and feed industries and the paper industry (Pedersen, 1991). Dung *et al.* (1991) screened xylanase producing bacteria from soil and water samples that produced five types of xylanases. An alkalophilic *Aeromonas* sp. No. 212 (Ohkoshi *et al.*, 1985) have been detected in the Institute of Physical and Chemical Research, Japan, which produced three types of xylanases.

There has been an increasing interest in applying xylanases in the pulp industry during recent years. Vikari *et al.* (1994) initially demonstrated the use of xylanases for the selective removal of hemicelluloses from kraft pulp prior to pulp bleaching. Since then, research has been performed on this subject. An *Aeromonas* strain from drain water have been isolated, that produces xylanase. So the main aim of this study is to search more active xylanase producing microorganisms and to isolate and to characterize the xylanase gene, which would be helpful to elucidate the mechanism of such xylanase activity.

Materials and Methods

Bacterial strain: For the screening of xylanase-producing bacteria, drain water samples which were collected from the Rajshahi University Campus and all around its local regions. *E. coli* LE 392 strain used in the transformation experiment was supplied by the Department of Biochemistry and Molecular Biology, Yamaguchi University, Japan.

Media and culture conditions: Nutrient agar media, MacConkey agar media and xylose lysine deoxycholate agar were used as a solid medium through out the work. Xylan agar (M-9 medium) plates were used for the isolation and identification of the suspected bacteria and the bacteria were cultured at 37°C.

Isolation and Characterization of bacteria: All the xylanase-producing bacterial strains which were isolated by their growth on xylan agar media as clear zones and xylanolytic properties were characterized according to the biochemical tests described in the "Bergey's Manual of Determinative Bacteriology".

Biochemical tests used to characterize the isolated strains

Catalase test: Catalase test was performed to determine the ability of the organism to liberate gas (O_2) from hydrogen peroxide (H_2O_2) by enzymatic (catalase) degradation.

Oxidase test: Oxidase is an enzyme generally found in aerobes. The redox dye, tetramethyl-para-phenyline-diamine-dihydrocholoride was reduced to a deep purple colour by the microorganism.

Urease test: The enzyme urease produced by certain bacteria is capable of decomposing urea to ammonia which causes alkaline reaction. Urease test requires Christensen's urea agar media containing peptone 1.0g, sodium chloride 5.0g, potassium dihydrogen phosphate 2.0g, glucose 1.0g, phenol 6.0ml and agar 20g.

Citrate utilization test: The test organism was cultured in a medium which contains sodium acetate, an ammonium salt and the indicator bromophenol blue.

Fermentation test: This test is used to differentiate the organisms that ferment carbohydrate. Oxidation fermentation medium containing peptone 2.0 g, sodium chloride 5.0 g, dipotassium hydrogen phosphate, bromothymol blue 3 ml and agar 2.5 g is used in the fermentation test of carbohydrates.

Antibiotic susceptibility: Bacterial strain resistant to antibiotic was tested by disc diffusion method (Bauer *et al.*, 1966) using cotrimoxazole, ampicillin and amoxycillin. Resistant strains isolated by disc diffusion method were further confirmed for their drug sensitivity by antibiotic spread plate method.

Measurement of xylanase activity: Assay mixture consisted of 0.5ml of enzyme solution and 0.5 ml of 1% oat spelt xylan in 50 mM sodium phosphate buffer, pH 7.0. After incubation at 50°C for 10 min, the reaction was terminated by adding 1ml of 3, 5-dinitrosalicylic acid. Xylanase activity was determined by

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detecting the release of reducing sugar from the substrate. The amount of reducing sugar present was determined by dinitrosalisylic acid method (Miller *et al.*, 1960). The enzyme activity was expressed as the amount of reducing sugar (xylose) released per ml of the sample.

Isolation of the multi-drug resistant bacteria: The multi-drug resistant *Aeromonas* strains were isolated from the selected strains containing of xylanolytic activity using the disc diffusion method of Bauer *et al.* (1966).

Plasmid DNA extraction and agarose gel electrophoresis: A single colony of the isolated multidrug resistant *Aeromonas* was inoculated into 100 ml LB broth containing 0.25 mg ml^{-1} of antibiotic solution in 250 ml conical flask and incubated at 37 °C overnight with constant shaking. This culture was then subjected for the extraction of plasmid DNA according to Holmes and Quigley (1981). The extracted plasmid DNA was then purified with polyethylene glycol (PEG) according to Maniatis *et al.* (1989). The purified plasmid DNA was then subjected to electrophoresis by using 0.8% agarose. Plasmid transfer to a sensitive *E. coli* LE 392 strain. Competent cells were prepared by calcium chloride procedure modified from Cohen *et al.* (1972). An *E. coli* LE 392 strain sensitive to amoxycillin, ampicillin and cotrimoxazole was inoculated in a 20 ml LB broth and grown for 8 hours at 37 ºC with slow shaking for the experiment. Transformation of the isolated plasmid DNA to the *E. coli* LE 392 was carried out according to Cohen *et al.* (1972).

Extraction of transformed plasmid DNA from *E. coli* LE 392: After the transformation experiment, plasmid DNA was extracted from the transformed *E. coli* LE 392 according to Holmes and Quigley (1981). The extracted plasmid DNA was purified and subjected to agarose gel electrophoresis.

Results

Bacterial strains were isolated from drain water. In a preliminary experiment of this research, two bacterial strains were spread on two different xylan agar plates for isolation and rapid identification of the xylanase-producing bacteria and xylanolytic properties. The plate was then incubated at 37°C for 48 h. The colonies which formed clear zone on the xylan agar plates were picked up and were then further purified by pure culture technique. The xylanolytic, clear and transparent colonies producing bacteria on the xylan agar plate were shown in Fig. 1.

Morphological and colonial characterization: Table 1 summarizes the morphological and colonial characteristics of isolates belonging to the genus *Aeromonas*. Colonies of isolates on nutrient agar and selective media were circular, convex, translucent and yellow or white. They grew in nutrient and KCN broth. They did not form brown water soluble pigment. All of the strains were gram negative, short rods.

Biochemical tests: All isolates were catalase and oxidase positive, and utilized mannitol, arginine and arabinose. They hydrolyzed starch and gelatin. No requirements for vitamins or other growth factors and fermentation of sucrose maltose and lactose were observed. The strains grew in peptone water without NaCl. Results of biochemical test have showed in the Table 2.

Identification of drug resistance gene in bacteria: After identification of bacterial samples were *Aeromonas* by catalase, oxidase and gram reaction, the bacterial strains were further studied for drug resistance gene in xylanase producing bacteria. In drug resistance test by disc diffusion method the bacterial samples showed multi-drug resistance. In this step, cotrimoxazole, ampicillin and amoxycillin resistant strains were isolated as multi-drug resistant strains (Table 3).

Plasmid profile of multi-drug resistant *Aeromonas*: Plasmid DNA was isolated from multiple drug resistant and xylanase producing *Aeromonas* bacterial sample according to the procedure described in materials and methods section. The plasmid profile showed that the selected strain of *Aeromonas* contains only one plasmid of about 30 kb in size which was detected by comparing to a marker DNA (Fig.1).

Drug resistance study of the transformed strain: To characterize the multi-drug resistance whether it is plasmid mediated or not and to identify the responsive plasmid coding multiple drug resistance in *Aeromonas* strain, the plasmid DNA was isolated from the strain and was transferred into sensitive *E. coli* LE392. *E. coli* LE392 was initially sensitive to at least six different conventional antibiotics like cotrimoxazole, ampicillin, amoxycillin, erythromycin, tetracycline and doxycyclin. Transformed colonies were isolated and tested by disc diffusion method using cotrimoxazole, ampicillin, amoxycillin, erythromycin, tetracycline, and doxycyclin discs. It was observed that *E. coli* LE392 that was found sensitive to the antibiotics used in the sensitivity test before transformation (Fig. 3) experiment became completely resistant to cotrimoxazole, ampicillin and amoxycillin after plasmid acquisition on both nutrient (Fig. 4) and xylan agar plate.
E. coli LE 392 transformants were picked up and stored. The multi-drug resistance was again confirmed by disc diffusion method using cotrimoxazole, ampicillin, amoxycillin, erythromycin, tetracycline and doxycyclin discs (Table 4). A clear zone was observed around the erythromycin, tetracycline and doxycyclin disc on both nutrient and xylan agar plate.

Xylanase activity study of the transformed strain: The xylanase encoding plasmid DNA in *Aeromonas* strain was isolated and was transferred into non-xylanase producing *E. coli* LE392, which was not produced clear zone on xylan plate after 48 h incubation at 37°C (used as control plate). In the transformation experiment, bacterial growth on the experimental and control plates were compared. Growth on experimental plates was noted but no growth on the control plate (Table 5).

Discussion

In this study, bacterial strains have been isolated from drain water with isolated, which degraded β-1, 4 xylans and belong to *Aeromonas* genus. In the primary step of this study, the xylanase-producing bacteria were isolated from the collected samples by screening procedure from their colonical growth as clear zone and xylanolytic properties on xylan agar plate. The xylanase activity in each strain was confirmed by measuring the amount of reducing sugars liberated from xylan by the DNS method (Miller, 1960) using crude xylan extract. After isolation, multi-drug resistance of these strains were determined by disc diffusion and antibiotic spread plate method. It was observed that, the strains were resistant to three antibiotics i. e. cotrimoxazole, ampicilline and amoxycillin and were sensitive to three antibiotics i. e. erythromycin, tetracycline and doxycyclin.
In the next step of this study, xylanase-encoding plasmid DNA from xylanase-producing and multi-drug resistant *Aeromonas* bacteria was isolated by Mini-Scale boiling method. The purified plasmid DNA was subjected to agarose gel electrophoresis with reference to a marker DNA (λDNA Hind III digested), a single plasmid of about 30 kb in size was measured. Khan *et al.* (2000) also reported a multi drug resistant gene (s) harboring on a 20 kb plasmid in *Salmonella typhi* that causes typloid.
To characterise the xylanase activity and drug resistance, the xylanase encoding plasmid DNA from xylanase producing and multi-drug resistant *Aeromonas* bacteria has transferred into non-xylanase producing and sensitive *E. coli* LE392. The plasmid DNA from transformed bacteria showed (Fig. 2) the presence of only one band of about 30 kb in size at the same position of 30 kb band of original strain. The transformed strains produced

Table 1: Morphological test of primary collected bacterial samples

Sample no.	Xylanolysis on xylan agar	Gram staining	Catalase test	Oxidase test	Comments
S-1	+	-	+	+	May be *Aeromonas*
S-2	+	-	+	+	May be *Aeromonas*

+ means Positive, - means Negative

Table 2: Biochemical tests for the identification of the bacterial strain

Sample no.	Motility test	Urease test	Citrate test	Indole test	Xylanase test	Fermentation test	Comments
S-1	+	-	-	+	+	+	*Aeromonas*
S-2	+	-	-	+	+	+	*Aeromonas*

+ means Positive, - means Negative

Table 3: Drug resistance of bacterial samples by disc diffusion method

	Diameter (mm) of zone of inhibition produced against			
Strain no.	Cotrimoxazole	Ampicillin	Amoxycillin	Comments
S-1	No zone	No zone	No zone	Resistant
S-2	No zone	No zone	No zone	Resistant
E.coli LE392	Clear zone	Clear zone	Clear zone	Sensitive

Table 4: Multi-drug resistance of transformed *E. coli* LE392 on both nutrient and xylan agar plate

Name of antibiotics	Conc. of antibiotic (μl/disc)	Diameter of clear zone (mm) on NA plate	Diameter of clear zone (mm) on XA plate	No. of drug resistant colonies appeared	Comments
Cotrimoxazole	30 units	---	---	Numerous	Resistant
Ampicillin	12 units	---	---	Numerous	Resistant
Amoxycillin	28units	---	---	Numerous	Resistant
Erythromycin	17 units	26	15	---	Sensitive
Tetracycline	32 units	23	8	---	Sensitive
Doxycyclin	34 units	24	14	---	Sensitive

Symbols: '--- Means no clear zone produced. NA=Nutrient agar, XA=Xylan agar

Table 5: Expression of xylanase in the transformant *E. coli* LE392

			No of transformed colonies appeared on the xylan agar plant		
Plasmid DNA source	Recipient strain	Plate made with xylan agar media	Experimental	Control	Remarks
		Plate-1	26	No colonies	Transformed
	E. coli	Plate-2	23	No colonies	Transformed
Aeromonas	LE392	Plate-3	19	No colonies	Transformed

Fig.1: Clear coloines of xyanase-producing bacteria on xylan agar plate after 48 h incubation at 37 °C

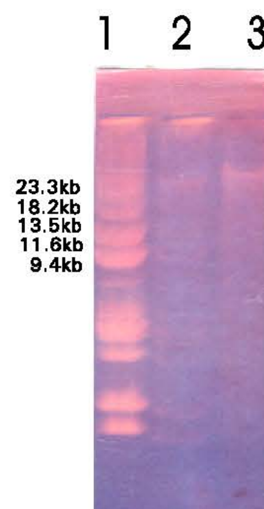

Fig.2: Plasmid profile of transformed strain *E.coli* LE 392. The plasmid DNA of isolated *Aeromonas* strain was shown in lane 3, the DNE of transformed strain *E. coli* LE392 was shown in lane 2 and the plasmid marker DNE in lane 1

clear zone on xylan agar plate. From this experiment it was observed that *E. coli* LE392 which was found non-xylan degrading

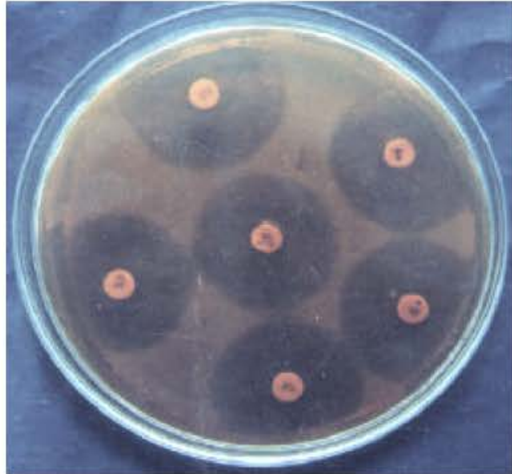

Fig. 3: Multi-drug sensitivity test of *E. coli* LE392 before transformation on nutrient agar plate. Drug used are cotrimoxazole, ampicilline, amoxycillin erythromycin, tetracycline and doxycyclin

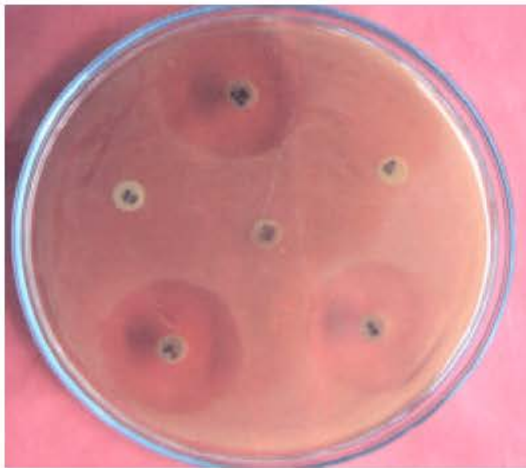

Fig. 4: Multi-drug sensitivity test of *E. coli* LE392 before transformation on nutrient agar plate. Drug used are cotrimoxazole, ampicilline, amoxycillin erythromycin, tetracycline and doxycyclin, TS= Cotrimoxazole, E= Erythroomycin, AP=Ampicillin, T=Tetracyclin, DXT=Doxycyclin

(do not produce clear zone on xylan agar plate) before transformation became xylan degrading due to xylanase encoding plasmid acquisition. This study indicated that xylanase gene was transformed from *Aeromonas* strain into non-xylanase producing *E. coli* LE392.

Further study is now going on to digest the plasmid DNA with different restriction enzymes, which will help to establish a complete restriction map of the plasmid. From this restriction map we may characterize the plasmid structure of the drug resistant *Aeromonas* strain. The result might help us to elucidate the molecular mechanism of multi-drug resistance in *Aeromonas* strain.

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