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Isolation and Molecular Characterization of Multidrug Resistance Gene Harboring on a 28 Kb Plasmid in *Aeromonas* sp.

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Abstract: *Aeromonas* strain is one of the predominant agents producing xylanase enzymes that are used for degrading the xylosidic linkages of xylan backbone in plant kingdom. *Aeromonas* strains were isolated from damp soil and straw at Rajshahi region. The strains were isolated on xylan agar media and screened by β-xylanolysis method. The isolated xylanase-producing strains were further tested by DNS (Dinitrosalisylic acid) method to confirm their xylanase activity. 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. *Aeromonas* strain also showed drug resistance to cotrimoxazole, ampicillin and amoxycillin and sensitive to erythromycin, tetracycline and doxycyclin. Plasmid DNA was isolated from the xylanase-producing multidrug resistant strain. Agarose gel electrophoresis of the isolated plasmid DNA showed the presence of a plasmid of about 28-Kb in size. The plasmid DNA was transformed into non-xylanase producing sensitive *E. coli* LE392. After transformation, it was found that the non-xylanase producing sensitive *E. coli* LE392 converted to xylanase-producing as well as resistant to cotrimoxazole, ampicillin and amoxycillin on both nutrient agar and xylan agar plate. Xylanase activity and multi-drug resistant in *Aeromonas* strain were proposed to be plasmid mediated. The genes were expressed simultaneously or co-operatively on both nutrient agar and xylan agar media.

Key words: Multidrug resistance, plasmid DNA, gene, xylanase, *Aeromonas*

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 α-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). Several of 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 (Vikari *et al.*, 1994). More recent reports showed that sugars i.e., xylose, xylobiose and xylooligomers can be prepared by the enzymatic hydrolysis of xylan (Patel, 1991). Whereas other sugar residues can be added using the transglycosylation activity of such enzymes as xylosidase. These xylose-containing sugars may be useful for research as well as for their rheological properties.

This study showed the isolation and molecular characterization of xylanase producing bacteria from damp soil and straw which degraded β-1, 4 xylans and belong to *Aeromonas* genus and also showed that the xylanase gene was localized in a 28 kb plasmid DNA. Xylanase-producing bacteria were suspected to have some drug resistance. This study makes a sense of urgency to study the mechanism of acquisition and transfer of xylanase gene as well as the genetic organization of the gene coding for resistance to cotrimoxazole, ampicillin, and amoxycillin. Therefore, the aim of this study was to isolate and to characterize the xylanase gene which would be helpful to elucidate the mechanism of such multi-drug resistance and xylanase gene transfer and also to design a new and more effective bacteria for proper production of large scale xylanase enzyme.

Materials and Methods

Bacterial strain: For the screening of xylanase-producing bacteria, damp soil and straw were collected from the Rajshahi University Campus during the month of August and September, 2001. *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.

Screening of the xylanase producing bacteria: 0.2 gm of damp soil and damp straw were dissolved in 100 ml sterile distilled water in two separate 100 ml volumetric flask and shacked vigorously. 0.2 ml of diluted sample solution was then spread on two different xylan agar plates for isolation and rapid identification of the xylanase producing bacteria from their colonical growth as clear zone and xylanolytic properties. The plates were then incubated at 37 °C for 48 hours (John *et al.*, 1994)

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.

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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 2.0g, sodium chloride 5.0g, dipotassium hydrogen phosphate, bromothymol blue 3ml and agar 2.5g is used in the fermentation test of carbohydrates.

Antibiotic susceptibility: Bacterial strain resistant to antibiotic was tested by disc diffusion method (Bauer *et al.*, 1996). Resistant strains isolated by disc diffusion method were further confirmed for their sensitivity by antibiotic spread plate method (Bauer *et al.*, 1996).

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

Multi-drug resistant in xylan-degrading bacteria: The multi-drug resistant *Aeromonas* strains were isolated from the selected strains containing of xylanolytic activity using the disc diffusion method. A 16 h broth cultures of the collected strains when grown at 37 °C and was spread on both nutrient agar plate and xylan agar plate using sterilized glass spreader. Then antibiotics were distributed on plates and kept the plates at 4°C for 4 h, so that the antibiotic can diffuse on the agar media. The plates were then incubated at 37 °C for 16 h and the growth of the bacteria was observed. The presence of a clear zone around the disc was the index of sensitivity to the antibiotic. The absence of such a clear zone or the presence of some colonies within the clear zone indicated that the collected strains were resistant to that antibiotic. The drug resistant bacteria tested by disc diffusion method were again confirmed by spreading its culture on the selected antibiotic plates of different concentrations. The plates were then incubated at 37°C and observed on next day. The clear plate indicated that the strains were sensitive to this selected concentration and presence of colonies on the plate indicated that the strains were resistant to that selective concentration.

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). Plasmid transfer to a sensitive *E. coli* LE392 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°C with slow shaking for the experiment. Transformation of the isolated plasmid DNA to the *E. coli* LE392 was carried out according to Cohen *et al.* (1972).

Transformation of multi-drug resistant xylanase gene into sensitive and non-xylanase producing *E. coli* LE392: Hundred µl of transformed competent cells were spread onto agar SOB medium containing 20 mM $MgSO_4$ and cotrimoxazole, ampicillin and amoxycillin on different selection plates. These selection plates made with antibiotics mentioned above to which the donor *Aeromonas* strains were resistant. Again 100 µl of transformed competent cells were spread onto xylan agar plates. These plates made with xylan agar media to which the donor *Aeromonas* strains produced clear zones. Hundred µl of competent cells from control tube was also spread on different selection antibiotic plates and xylan agar plates respectively. The plates were left at room temperature until the liquid has been absorbed. Then the plates were incubated at 37 °C for 24-36 h and observed the plates for the appearance of the drug resistant and xylanase-producing bacteria.

Xylanase activity of the transformants: A 16 h xylan-broth culture of the transformants was grown at 37 °C and spread on xylan agar plates using sterilized glass spreader. These plates were made with xylan agar media to which the transformants produced clear zone. The plates were then incubated at 37 °C for 48 h and observed the plates for the appearance of the transformants. The clear plate (no growth) indicated that the transformants were non-xylanase producing and presence of clear zone around the colony indicated that the transformants were xylanase producing.

Results

Isolation and characterization of xylanase-producing bacteria: For a preliminary experiment of this study, bacterial samples were collected from damp straw and damp soil. β-xylanolytic clear and transparent zone (on the xylan agar plate) producing bacterial strains were collected according to the method described in the materials and methods section. When β-xylanolysis was observed on the xylan agar plate after 36 to 48 h incubation at 37 °C (Fig. 1), the β-xylanolytic bacterial strains were collected and further purified by pure culture technique. Isolated bacterial colonies from different sources were primarily screened by staining method, microscopic examination, oxidase and catalase test. The results of various physicochemical tests of the isolated xylanase-producing *Aeromonas* strains have shown in the Table 1.

Table 1: Biochemical and physiological characteristics of the isolated strain, *Aeromonas*

Characteristics	Result
Growth in nutrient broth at 37°C	+
Growth in KCN broth	+
Fermentation of mannitol and lactose	+
Gram staining	+
Xylanase activity	+
Indole	+
Oxidase	+
Catalase	+
Urease	+
Citrate utilized	--
Gas from glucose	--
Growth in peptone water without NaCl	+
Motility	+

Table 2: Drug resistance of three bacterial samples by disc diffusion method

Strain no.	Cotrimoxazol (15µl/disc)	Ampicillin (15µl/disc)	Amoxycillin (15µl/disc)	Comments
1	No zone	No zone	No zone	Resistant
2	No zone	No zone	No zone	Resistant
3	No zone	No zone	No zone	Resistant
4	Clear zone	Clear zone	Clear zone	Sensitive

Identification of drug resistance gene in xylanase-producing bacteria: The *Aeromonas* strains were further studied for drug resistance to isolate the multi-drug resistant bacteria. In drug resistant test by disc diffusion method the strains showed multi-drug resistance. In this step, cotrimoxazole, ampicilline and amoxycillin resistant strains were isolated as multi-drug resistant strains (Table 2).

Table 3: 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	25 units	---	---	Numerous	Resistant
Ampicillin	10 units	---	---	Numerous	Resistant
Erythromycin	15 units	22	13	---	Sensitive
Tetracycline	30 units	23	6	---	Sensitive
Doxycyclin	30 units	25	12	---	Sensitive

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

Fig.1: Xylanolysis colonies on xylan agar plate are surrounded by a clear, colourless zone after 48 h incubation at 37 °C

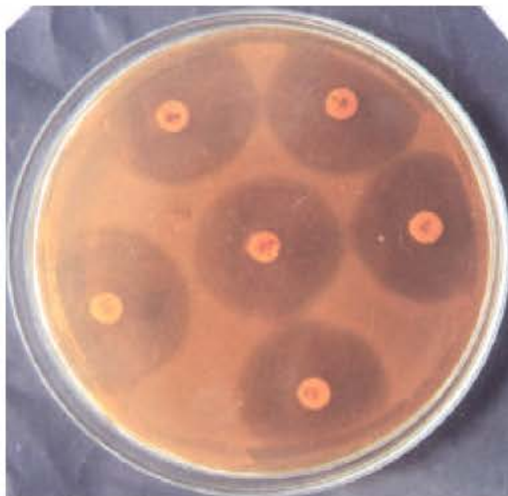

Fig.2: Multi-drug sensitivity test of *E. coli* LE392 before transformation on nutrient agar plate, TS = Cotrimoxazole, E = Erythroomycin, AP = Ampicillin, T = Tetracyclin, DXT = Doxycyclin

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 (Fig. 2) to at least six different conventional antibiotics like cotrimoxazole, ampicillin, amoxycillin, erythromycin, tetracycline, and doxycyclin.

Transformed colonies were isolated and tested by disc diffusion

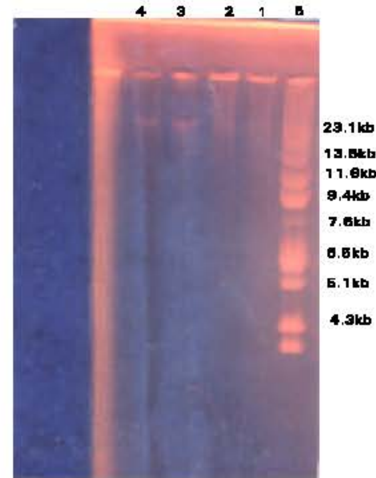

Fig.3: Plasmid profile of transformed *E. coli* LE 392. The DNA of transformed *E. coli* LE 392 was shown in lane 3 and lane 4 and the plasmid DNA of isolated *Aeromonas* strain was shown in lane 1 and lane 2 and the marker DNA (λ DNA Hind III digested) in lane 5

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 experiment (Fig. 2) became completely resistant to cotrimoxazole, ampicillin and amoxycillin after plasmid acquisition on both nutrient agar and xylan agar plate (figure not shown). But a clear zone was observed around the erythromycin, tetracycline and doxycyclin disc on both nutrient and xylan agar plate (Table 3).

Plasmid profile of transformed *E. coli* LE392 and donor *Aeromonas* strain: An attempt was taken to transfer plasmid DNA from multi-drug resistant *Aeromonas* to a sensitive *E. coli* LE392. This attempt was totally successful. The plasmid DNA isolated from transformed *E. coli* LE392 by boiling method described in materials and methods. After electrophoresis, the gel was stained in ethidium bromide solution (0.5 μg/ml) for 20 min. Then the gel was washed with tap water and placed on an UV-transilluminator and finally photographed by a camera. Plasmid profile showed that (Fig. 3) plasmid DNA from transformed *E. coli* LE392 and plasmid DNA of donor *Aeromonas* strain were same size I. e., about 28 kb in size. This plasmid DNA band corresponding to that of original strain is the indication of successful transformation. This transformation study indicated that multiple-drug resistance and xylanase activity in the selected *Aeromonas* strain might be due to the presence of 28 kb plasmid DNA.

Discussion

In this study the bacterial strains were isolated from damp soil and straw, which degraded β-1, 4 xylans and to 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 dinitrosalisylic acid method (Miller, 1960) using crude extract. After isolation of xylanase-producing strains, the strains was identified as *Aeromonas* on the basis of catalase, oxidase, gram-staining reaction, morphological and cultural characteristics and different biochemical tests. Under microscope the cells were arranged in singly, in pairs or chains, the cells were straight, rod shaped with rounded ends to coccoid. They were motile and gram-negative in staining reaction. They grew well on simple laboratory medium in the temperature ranges from 35-41°C and optimally at 37 °C. 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.

The 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 28 kb in size was measured (Fig. 3). After transformation experiment, the transformed strains appeared on plates were tested for their xylanolytic activity by their colonical growth as clear zone on xylan agar plate after 48 h at 37 °C. From this experiment it was observed that *E. coli* LE392 which was found non-xylan degrading (do not produce clear zone on xylan agar plate) before transformation became xylan degrading (produce clear zone on xylan agar plate) due to xylanase-encoding plasmid acquisition. This xylanase activity study indicated that xylanase gene was transformed from *Aeromonas* strain into non-xylanase-producing *E. coli* LE392. The transformed strains appeared on plates were tested for their multi-drug resistance by disc diffusion method on both nutrient agar and xylan agar plates. From this experiment it was observed that *E. coli* LE392 which was sensitive to cotrimoxazole, ampicilline and amoxycillin before transformation became resistant to these antibiotic due to this plasmid acquisition. These strains again tested by spread method using 30, 40 and 60 μg/ml of cotrimoxazole plates on which 20, 17 and 12 drug resistant colonies were appeared respectively. Similarly, in case of ampicillin and amoxycillin using same concentration plates; 18, 12 and 7 and 19, 13 and 10 drug resistant colonies were appeared on the respective plates respectively. But no drug resistant colonies were appeared on the control plates. This drug resistant study indicated that cotrimoxazole, ampicillin and amoxycillin resistance was transformed from *Aeromonas* strain into *E. coli* LE392. This result indicated that plasmid of 28 kb in size was transferred. Transformation study showed that xylanase activity and multi-drug resistance in *Aeromonas* strain from damp soil and damp straw is due to the presence of extrachromosomal DNA or plasmid present in the bacteria. Toshiaki Kudo, Riken Institute, Japan (1985), reported that plasmid pAX1 from *Aeromonas* sp. No 212 was isolated from transformants producing xylanase, and the xylanase gene was located in a 6.0 kb HindIII fragment. Gilbert (1988) reported that a 6 kb DNA fragment was found that encodes 5 open reading frames encoding a xylanase, a β-xylosidase an acetyl-esterase and two ORF with unknown functions in *Caldocellum saccharilyticum*.

It should be noted that due to limitation of our laboratory facilities, it was not possible to characterize the plasmid in details. Further study is now going on to digest the plasmid DNA with different restriction enzymes, which will help us to establish a complete restriction map of the plasmid. From this restriction map we may characterize the plasmid structure of the xylanase-producing *Aeromonas*. Site-directed or random mutagenesis could also be carried out to construct xylanase over expressing strains. The result might helps us to elucidate the molecular mechanism of xylanase activity in *Aeromonas* strain and to develop new and more effective bacteria for large scale production of xylanase enzyme for industrial use.

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