Global Journal of Plant Ecophysiology, 3(1): 25-31, 2013
ISSN 2074-0891
This is a refereed journal and all articles are professionally screened and reviewed

ORIGINAL ARTICLES

Screening, Isolation and Identification of cellulolytic *Bacillus* isolates from Soil and Compost

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ABSTRACT

Fifty five *Bacillus* isolates were screened using morphological tests, biochemical and molecular characterization using 16s rDNA analysis , isolated from compost, and alkalian silty clay soil (rhizosphere of potato plant) in Shambat, Khartoum.North, Sudan. Screening of cellulase producing isolates was done using carboxyl methyl cellulose (CMC) as a substrate at 25°C. Twenty six isolates with cellulase activity were found to be cellulase producers. Among the isolates, four isolates, 9+, 23, 20 and 13 showed high potential on producing extracellular cellulase. Genetic distance between the four isolates with high cellulase activity was determind with RAPD analysis using OPC-3 primer. Conventional isolation and identification is the most accurate method for detection of an active organism in environmental samples . However, this method is tedious laborious and time consuming. The PCR provides a reliable, rapid, sensitive and specific assay for monitoring *Bacillus* isolates. These results indicated that PCR using 16s r DNA and OPC-3 primer should be used as a routine technique for rapid identification of *Bacillus* isolates producing extracellular cellulase.

Key words:

Introduction

Over the years, culturable , cellulase-producing bacteria have been isolated from a variety of sources such as composting heaps, decaying plant material from forestry or agricultural waste, the feces of ruminants such as cows, soil and organic matter, and extreme environments like hot –springs, to name a few (Doi, 2008). Screening for cellulase producers can be done by enrichment growth on microcrystalline cellulose as a sole source of carbon, followed by the extraction of 16s rDNA\RNA to determine the molecular community structure of the environment and analyze whether families containing cellulase-producing species are present (Rastogi *et al.*2009). Screening for cellulase activity in microbial isolates is typically performed on carboxy methyl cellulose (CMC) containing plates (Hankin and Anagnostaki, 1977). However, plate –Screening methods using dyes are not qualitative or sensitive enough due to poor correlation between enzyme activity and halo size (Wittrup and Bailey 1988). Recently, Kasana and colleagues (2008) found that Gram's iodine for plate flooding in place of hexadecyltrimethyl ammonium bromide or congo red, gave a more rapid and highly discernable results.

The genus *Bacillus* includes a variety of phenotypically heterogeneous species exhibiting a wide range of nutritional requirement, physiological and metabolic diversities, and DNA base compositions (Kim *et al*. 2010). Morphological and physiological characteristics have traditionally provided a wealth of *Bacillus* systematic information for establishing Bacillus classification systems (Qingming *et al.* 1997). Advances have been made in automating and minimizing the detection times using biochemical methods, however biochemical identification is not accurate for determining the genotypic differences of microorganisms (Woese *et al*. 1985). A more accurate method for genotype determination is that of the molecular biological approach of ribotyping by comparing similarities in the r RNA gene sequences (Barney *et al*. 2001). Partial 16 s r RNA gene sequences and r RNA gene restriction patterns have been used for the rapid identification and classification of *Bacillus sp.,* and related genera (Kim *et al*. 2010).

In the present investigation, Screening for *Bacillus* isolates producing cellulase was done using CMC plate assay and Grams iodine. For the identification and differentiation of the isolates 16s r DNA analysis, RAPD analysis, morphological and biochemical tests were used.

Materials And Methods

Isolation of Bacillus isolates:

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Soil samples were taken from Shambat agricultural area. Khartoum North, Sudan (rhizosphere of potato plant), the soil was alkaline (pH8.5) and it was silty clay. Compost samples were taken from Shambat (depth 20cm). All samples consisted of ten sub-samples, taken randomly to obtain a representative samples. Isolation of *Bacillus* was carried out according to Lindquist (2006). Ten grams from each sample were taken and dissolved in 90 ml distilled water. Soil suspension was heated at 80°C for 15 minutes. After appropriate dilutions with sterile water, 0.1 ml of heated dilutions was spread on nutrient agar plates and incubated aerobically at 37°C for 24 hours, and then colonies were purified and prepared for testing.

Conventional identification of Bacillus isolates:

The isolates were morphologically and physiologically identified up to genus level according to Bergy's manual for bacteriology and Harrigan and Mac-Cance (1976). The purified colonies (11 colonies from soil sample, 15 colonies from compost) were subjected to the identification test shows in Table.1 and Table.2.

Screening of Bacillus isolates producing cellulase:

Screening of extracellular cellulase producing *Bacillus* was carried out according to Kasing (1995) with a modification from Kasana *et al*. (2008). Carboxymethylcellulose (CMC) agar was made with 0.5g carboxy methyl cellulose (a soluble from of cellulose) 0.1g $NaNO_3$, 0.1g $K_2 HPO_4$, 0.1g KCl, 0.05g $Mg SO_4$, 0.05g yeast extract and 0.1g glucose in 100 mL of water. The medium was solidified using 1.7% w/v agar. Borer (diameter 5mm) was used to make a well in the agar. Into the appropriate well 0.2 mL of each microbial culture was placed using sterile syringe. The plates were incubated for 10 days at 25-30C° then plates were flooded with Gram's iodine.

Cellulase production and preparation in liquid medium:

The enzyme was produced from the purified isolates that showed a positive reaction on CMC plates test. Cellulase production was done according to Kotchoni and Shonukan (2003) with slight modifications. A single fresh colony of each isolates was inoculated in nutrient broth medium for 24h at 37C° with Shaking at 150 rpm as preculture. The preculture was inoculated (1:20) in 100 mL medium, containing (per liter) 0.2% glycerol as carbon source, 0.2% carboxyl methyl cellulose as substrates for cellulase synthesis, 10g peptone, 1g K_2HPO_4 , 0.75g Mg SO4, 0.75g NaCl in a 0.1M KH_2PO_4 buffer pH6.0 in a conical flask volume 250 mL. Incubation was continued for 24, 48, 72, 96, 120 or 144 hours to determine the optimum time of inocubation at 25°C with shaking at 150 rpm. After each period of incubation the yield of cellulase was estimated in extracellular fluid after removal of bacterial cells from the culture broth by centrifugation at 500 rpm for 15minutes.

Cellulase assay:

Cellulase activity was determined by the method of Miller(1959) the assay mixture contained 0.5 mL of 2% carboxy methyl cellulose in 0.05 M Na – citrate buffer of pH 4.8 and 0.5mL of crude enzyme . The reaction was performed at 50°C for 30 minutes and stopped by addition of 3mL of 3.5-dinitrosalicylic reagent. The enzyme activity was obtained from a calibration curve prepared by the same procedure with D-glucose as the standard. One unit of enzyme activity was defined as the amount of enzyme required for liberating 1µ M of glucose per milliliter per minute and was expressed as µ M ml/ min. In accord with the International Union of Biochemistry one enzyme unit equals 1 micromole of substrate hydrolyzed per minute. For cellulase this was based on bonds hydrolyzed, that is micromoles of glucose released per minute. One micromole of glucose equals 0.180 mg. For a 30 minutes assay 1 mg of glucose equals 0.185 unit calculated as follows:

$$1 \text{ mg glucose} = \frac{1}{30 \times 0.18}$$

The enzyme unit (U) was calculated from the obtained mgs glucose.
U= mg glucose×0.185

Extraction of Bacillus chromosomal DNA:

DNA extraction from *Bacillus* isolates was done according to the boiling –centrifugation method (Soumet *et al*. 1994). A single colony was grown over night at 37°C in each 5 mL Luria –Bertani broth (containing; 10 g tryptone, 5.0 g yeast extract and 10 g Na CL) per liter distilled water (pH 7.5). Bacterial cells were precipitated and supernatant was discarded. The pellets were resuspended in 1mL de ionized distilled water;

the suspension was boiled for 10 minutes in a water bath and then immediately cooled on ice. The presence or absence of DNA was tested by electrophoresis on agarose gel and visualized under UV. Extracted DNA was then stored refrigerated until used as a template for PCR amplification.

Analysis of 16s rDNA:

The chromosomal DNA was used as a template for amplification of 16s rDNA via the polymerase chain reaction (PCR).The primers used were FW (5- GAG TTT GAT CCT GGC TCA G-3) as a forward primer and RV (5-AGA AAG GAG GTG ATC CAG CC-3) as reverse, the primer pair obtained from metabion international AG, Lena-Christ-Street 44/1, D-82152Martinsried-Germany. The PCR reaction mixture was composed of 4 µl of 5X FIRE POL PCR Master Mix (5X reaction buffer, 0.1% W/V Tween 20, 12.5 mM Mg CL_2, 1 mM d NTPs, 1 U Tag polymerase), 1 µl of each primer, 5 µL of template DNA and the volume was made up to 20 µl using sterile dd H_2O. The reaction mixture was incubated in a thermocycler (Techne/ flexigene. Cambridge, UK.) programmed to run 35 cycles repeatedly (Denaturation at 95°C for 5 min, primer annealing at 55°C for 1 minute and extension at 72°C for 1 minute, the final cycle included extension for 5 minutes at 72°C to ensure full extension of the products. The resulting PCR product were analyzed on 1.2% agarose gel (sigma) at 75 V, 400, stained in 0.5 µL/ml ethidium bromide for 1 hour and the gel was visualized in a gel documentation system (Biosystematica, MO 33874. Power 25 W) and photographed (digital camera, Nikon, 45000).

RAPD-PCR for the chosen isolates with high cellulase activity:

Classification of the chosen four isolates; 13, 9+, 20 and 23 with high cellulase activity, at the molecular level was done using RAPD Primer OPC-3 (5-CAC TGG CCC A-3) (metabion international AG, Lena-Christ-Street 44/1, D-82152Martinsried- Germany) RAPD assays were performed in 20 µl reaction mixture contained (Final concentration): 4µl of: 5X FIRE PoL PCR Master Mix (5 X reaction buffer, 0.1% W\V Tween 20, 12.5 mM Mg CL_2, 1 mM d NTPs, 1 U Taq polymerase), 1 µl of OPC-3 primer, 5 µl of template DNA and the volume was made up to 20 µl using sterile dd H2O. The RAPD reaction was run for 35 cycles in a DNA thermal Cycler (Techne/ flexigene). The following thermal profile was used for the PCR: denaturation at 95°C for 30 seconds, primer annealing at 35°C for 1 minute and extension at 72°C for 2 minutes. The final cycle included extension for 5 minutes. RAPD reaction products were electrophoresed as mentioned previously.

Resalts And Disscution

Conventional identification of the Bacillus isolates:

All isolates were found to be motile, Gram positive, rod-shaped and endo-spores forming. The characteristics shown by all of the isolates suggested that they belong to the family *Bacillaceae* according to Bergey's Manual of Systematic Bacteriology (Sneath and Mair. 1986). Results of biochemical characterization are shown in Table 1 and Table 2. Isolate 7 according to Bergey's Manual of Systematic Bacteriology (Sneath and Mair.1986) showed similarity to *Bacillus lichen forms*.

Table1: Biochemical tests for *Bacillus* isolated from compost

isolate	Gram	Oxidase	Catalase	Endospore	VP	Citrate	Gelatin	Starch	Growth at pH		Indole	NR	OF		Motility	Methyl red	NaCl			
									6.8	5.7			P	P-			2%	5%	7%	10%
16+	+	+	+	Central	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+
15+	+	+	+	Central	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20+	+	+	+	Central	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
9+	+	+	+	Central	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
10+	+	+	+	Central	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
13+	+	+	+	Subterminal	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7+	+	+	+	Central	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18+	+	+	+	Terminal	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+

21+	+	+	+	Central	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
22+	+	+	-	Subterminal	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
24+	+	+	+	Central	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
31+	+	+	+	Central	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
32+	+	+	+	Central	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
33+	+	+	+	Terminal	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
35+	+	+	+	Central	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+

NR=nitrate reduction
P=parafinin the tubes
P-=tubes without parafin
OF=oxidation fermentation

Table 2: Biochemical tests for *Bacillus* isolated from soil.

isolates	Gram	Oxidase	Catalase	Indospore	VP	Citrate	Gelatin	Starch	Growth at pH		Indole	NR	OF		Motility	Methyl red	Nacl			
									6.8	5.7			P	P–			2%	5%	7%	10%
7	+	+	+	Central	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	-
10	+	+	+	Subterminal	+	-	+	+	+	+	+	+	-	-	+	-	+	+	+	-
6	+	+	+	Central	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-
13	+	+	+	Central	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
22	+	+	+	Subterminal	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+	+
14	+	+	+	Central	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	-
18	+	+	+	Subterminal	+	+	+	+	+	+	-	+	+	+	+	-	+	+	-	-
23	+	+	+	Subterminal	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+
16	+	+	+	Subterminal	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+
20	+	+	-	Subterminal	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
17	+	+	+	Subterminal	+	-	+	+	+	+	-	+	+	+	+	-	+	+	-	-

NR=nitrate reduction
P=parafin in the tubes
P– =tubes without paraffin
OF=oxidation fermentation

Screening of Bacillus isolates producing cellulase:

All isolates subjected to carboxyl methyl cellulose plates assay were showed a positive result to the test by giving a sharp and distinct zone around the cellulase producing isolates .Gram's iodine formed a bluish black complex with cellulose but not with hydrolyzed cellulose, giving a sharp and distinct zone around the cellulose-producing microbial colonies within 3 to 5 minutes. This new method is rapid and efficient; there fore, it can be easily performed for screening large number of microbial cultures of both bacteria and fungi (Kasana *et al*. 2008). Since *Bacillus* is motile, screening methods using dyes are not qualitative or sensitive enough due to poor correlation between enzyme activity and halo size. Cellulase from *Bacillus* isolates was produced in liquid production medium as a more accurate, quantitative method to support CMC plates assay for use in screening for *Bacillus* producing cellulase. The cellulase activities of isolates ranged between 0.799 Unit/ml (for isolate16+) to 3.48 Unit/ml (for isolate13) Table3. shows the results. Among the tested isolates, four isolates 9+, 23, 13 and 20

showed high potential in cellulase production and had an average cellulase activity of 2.89, 3.124, 3.48 and 3.53 Unit/ml, respectively.

Table 3: Cellulase activity and optimum time of incubation

Isolates	Source	Cellulase activity U/ml	Optimum time of incubation (hour)
24+	Compost	2.96	120
16	Soil	2.04	144
16+	Compost	0.79	120
9+	Compost	2.89	120
32+	Compost	2.66	96
33+	Compost	1.54	144
20+	Compost	2.23	96
22+	Compost	1.56	144
23	Soil	3.12	120
31+	Compost	1.50	144
18	Soil	1.54	144
35+	Compost	1.27	48
15+	Compost	2.36	120
21+	Compost	1.93	120
13+	Compost	2.52	144
20	Soil	3.53	72
7+	Compost	1.75	48
10+	Compost	2.82	96
18+	Compost	2.20	144
7	Soil	2.69	144
10	Soil	1.99	72
6	Soil	2.43	120
13	Soil	3.48	144
22	Soil	1.28	144
14	Soil	3.13	96
17	Soil	2.48	144

Dendrogram using Average Linkage (Between Groups) Rescaled Distance Cluster Combine

Fig. 1: Cluster analysis of DGGE profiles of *Bacillus* isolates from compost (+) and cow dung (-) genomes ith 16srDNA primer. Similarity was analyzed using SPSS clustering.

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identification of Bacillus isolates:

Molecular:

The results of denaturing gradient gel electrophoresis (DGGE) analysis of 16s rDNA PCR fragments showed degree of similarity among the isolates, isolates 13 and 23 were closest to each other and showed similar banding pattern. For analysis only the scorable bands were included and every single band was considered as a single locus/ allele for all the genetic analyses. The total numbers of loci traced by this primer was 109. The usefulness of 16s rDNA sequence analysis in the identification of conventionally unidentifiable isolates has not been evaluated with a large collection of isolates (Drancourt *et al*. 2000). Fig1. and Fig2. show cluster analysis of DGGE profiles.Genetic distances for the four selected isolates with high cellulase activity based on the 25 bands of the OPC-3primer obtained from the results of RAPD revealed two groups, among which isolate 13 and 23 were most close to each other results are shows in Fig3 and Fig4 . Since RAPD are primed from random region of the genome, it produces fingerprint of different loci from the entire genome (Qingming *et al*. 1997). Similarity according to the biochemical tests and 16S r DNA analysis agrees with genetic distance.

Dendrogram using Average Linkage (Between Groups) Rescaled Distance Cluster Combine

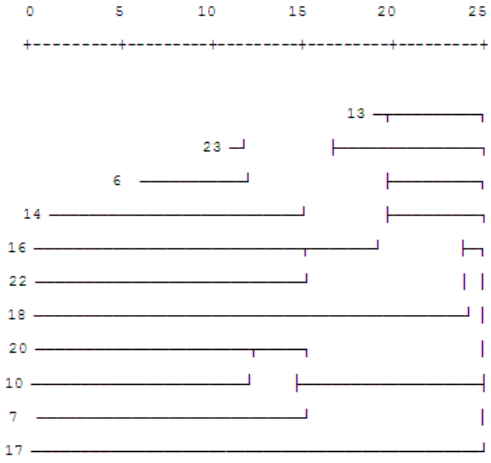

Fig. 2: Cluster analysis of DGGE profiles of *Bacillus* isolates- from soil- genomes with 16s rDNA primer. Similarity was analyzed using SPSS clustering.

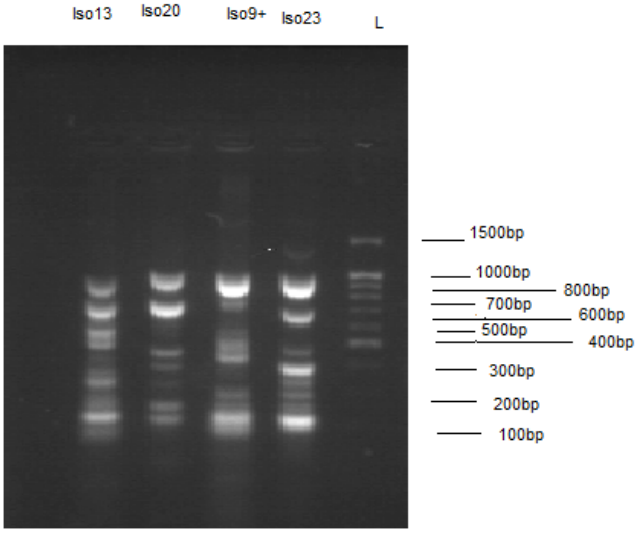

Fig. 3: RAPD patterns amplification of chosen isolates iso13, iso20, iso9+, and iso 23 genomes with OPC-3 primer.

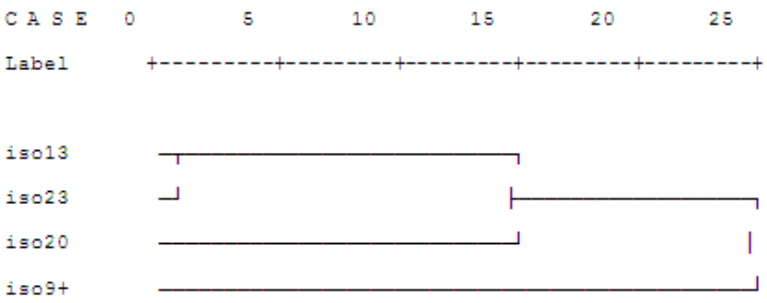

Fig. 4: RAPD analysis carried out with primer OPC-3 on the genome of chosen isolates (iso13, iso23, iso20, and iso9+) with OPC-3 primer. Similarity was analyzed using SPSS clustering.

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