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ORIGINAL RESEARCH

Molecular Characterization of Microorganisms in Municipal Solid Waste for Production of Industrial Enzymes and Enhanced Biodegradation

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ABSTRACT
Increase in volume of waste generated by municipal residents, change in the quality of waste composition and the treatment and disposal method of waste collected are of major concern. The exploitation of the metabolic versatility of microorganisms is advantageous in biological waste treatment but the actual number of degraders of a target compound in a mixed culture may only represent 5-10% of the microbial community. To understand how microorganisms may be manipulated and exploited to reduce the frequency of such breakdowns and shorten start-up times of biological waste treatment, the important bacterial strains actively involved in the degradation of municipal solid waste were isolated, screened and enzyme assays were performed. The identified strains were then used for molecular studies by DNA isolation and 16S rRNA PCR amplification. The molecular characteristic study was done by sequence analysis and phylogenetic tree construction. It was concluded that *Cellulomonas fimi*, *Bacillus thuringiensis*, *Paenibacillus favisporus*, *Lysinibacillus*, *Bacillus tequilenis, Geobacillus thermoleovorans*, *Micrococcus luteus*, *Bacillus subtillis*, *Bacillus halotolerans*, *Escherichia coli*, *Bacillus sp.*, *Paenibacillus polymyxa*, *Bacillus cereus*, *Paenibacillus mucilaginosus, Ruminococcus champanellens*, *Serratia marcescens*, *Ruminococcus albus*, *Pseudomonas stutzeri* are the most dominant species in municipal solid waste and proved effective in the degradation of the kitchen wastes. We therefore developed an efficient bacterial consortia that could concomitantly degrade different components of the organic wastes with the help of their enzymes in less span of time under natural conditions without producing any foul odour.

KEY WORDS: *Municipal Solid Waste, Enzymes, Molecular Characterization, Bacteral Consortia, Phylogenetic tree.*

INTRODUCTION

Municipal solid waste is a major problem faced by the people worldwide. The estimated quantity of Municipal Solid Waste (MSW) generated worldwide is 1.7-1.9 billion metric tons (Chalmin & Gaillochet (2009). Proper handling and management of solid waste is a biggest challenge to present society. Improper handling and dumping of waste in public areas cause severe environmental and health issues. With the rise in world population the volume of waste and disposal of waste has become a major problem. Economic development, urbanization and improved living standards in cities increase the quantity and complexity of generated solid waste. If not managed properly, it leads to degradation of urban environment, puts strain on natural resources and leads to health problems (Kumar et al., 2009). Landfills represent an environmentally acceptable disposal method of municipal solid waste on ground. Solid wastes are generated

from various domestic and commercial areas like hospitals, school, office, houses, hotels, restaurants etc. Solid waste includes both biodegradable (30-55%) and non biodegradable components (40-45%) and recyclable mater (5-10%). India produces approximately 40 billion municipal wastes yearly (Joshi & Ahmed 2016).

At the present, the most widely used waste disposal method is land filling, that cause major pollution and lose of potentially valuable materials that can be processed as fertilizer, fuel and fodder. The organic fraction of waste include about 75% sugar and hemicelluloses, 9% cellulose, and 5% lignin , carbohydrate, amino-acids, peptides and protein, volatile acids, fatty acids and their esters. Municipal waste degradation is carried out by different strains of bacteria and fungi. Microbial strains that produce different enzymes that aid in degrading the solid waste. Microbial degradation of solid waste depends upon temperature, pH, moisture and substrate composition. Microorganism performs their metabolic processes rapidly and with remarkable specificity under ambient conditions, catalysed by their diverse enzyme-mediated reactions. Waste generation and its control have taken an important role in our environment. With the doubling of population and changing lifestyle pattern of the inhabitants the quantity of municipal waste generated is increasing in an alarming rate. Most of this waste is subjected to dumping in a specified disposal yard. The greatest challenge to the environmentalists is the eco friendly management of this waste and application of microorganisms in this context has got an age over other available technologies.

Organic waste is consumed by the bacteria, used as nutrients by the bacteria, and is no longer present to produce odours, sludge, pollution or unsightly mess. When bacteria consume waste, they convert the waste into safe by products and in due course of this conversion they actually produce several metabolites to break down the complex waste into simple compounds. Soil microorganisms are increasingly becoming an important source in the search for industrially important molecules. Extent of microbial diversity in nature is still largely unknown, thus there might be many more useful products yet to be identified from soil microorganisms (Alexander M (1977). In soil 80 to 99% of microorganisms remain unidentified whereas these biological communities are known to play a dominant role in maintaining a sustainable biosphere (Saha & Santra (2014).

Today both academic and industrial interest in soil bacteria is on the rise, in search of deriving these unique biologically active metabolites and novel commercially important products from them. There is an immense possibility to screen effective bacterial strains from waste dump sites with valuable applications. To cope up with the demand for new organisms with properties of production of unique enzymes/molecules for industrial application and waste degradation there have been a constant effort in isolating novel bacteria from diverse environment.

Bacteria use wastes for their own metabolism and finally they produce some simple and useful compounds which are important for soil health, plant growing and over all to keep well balance of natural ecosystem. Bacteria along with saprobic fungi are an important contributor to optimal agricultural and kitchen wastes bioconversion. Unscientific disposal causes an adverse impact on all components of the environment and human health. Microorganism performs their metabolic processes rapidly and with remarkable specificity under ambient conditions, catalysed by their diverse enzyme-mediated reactions (Adrio & Demain 2014).

RESULTS AND DISCUSSION

Isolation, identification and maintenance of bacterial isolates

The municipal waste was used as the sample to find out bacteria with efficient ability to degrade kitchen waste. A total of 150 isolates were isolated by serial dilution method. The microbes were then made pure culture by growing on nutrient agar medium. The identification was based on colony characteristics, biochemical tests and molecular characterization. The identified isolates were then stored in storage tube at 4^{o}C. The morphology of colony was observed on simple nutrient agar plate. The bacterial colonies were irregular in shape. Colony morphology was observed on Nutrient agar. The colonies were further purified on agar by morphology.

Table 1: Characteristics of the twenty isolates

Isolates	Colony colour	Colony nature	Colony shape	Gram staining
T2	Cream	Irregular	Rods	Gram negative
T3	Cream	Mucoid	Rod	Gram positive
T4	Cream	Rhizoid	Rod	Gram positive
TT5	Off white	Rhizoid	Rods	Gram positive
TT7	Off white	Rhizoid	Rods	Gram positive
M4	Whitish cream	Spherical	Rods	Gram positive
M5	Cream	Mucoid	Rods	Gram negative
M6	Whitish cream	Rhizoid	Rods	Gram positive
Wm7	Off white	Irregular	Rods	Gram positive
Wm8	Whitish cream	Mucoid	Rods	Gram positive
P1	Off white	Rhizoid	Rods	Gram positive
P2	Off white	Irregular	Rods	Gram positive
L4	Off white	Rhizoid	Rods	Gram positive
L6	Opaque	Round	Rods	Gram negative
	Off white	Mucoid	Rods	Gram positive
Bb4	Off white	Rhizoid	Rods	Gram positive
Mu12	Off white	Rhizoid	Rods	Gram positive
Bw1	Off white	Irregular	Rods	Gram positive
Bw2	Off white	Rhizoid	Rods	Gram positive
Bw8	Off white	Rhizoid	Rods	Gram positive

Extracellular enzyme production

All the isolated bacterial strains were screened qualitatively for the production of five important enzymes. The strains were individually inoculated by single streaking on selective media such as starch agar (2% starch), Skim milk agar, CMC agar, pectate agar and Basal salt media with 1% tributyrin to isolate amylase, protease, cellulase, pectinase, lipase producers respectively. The Petri plates were incubated overnight at either room temperature or 37°C. The plates were then flooded with indicator solution and the development of clear zone around the growth of organism was considered positive for enzyme activity. The microbial strain that shows a zone of clearance of 25mm were chosen

for the quantitative assay methods. The amount of enzyme produced was measured quantitatively by spectrophotometric method. Similar study was reported by Ramesh & Mathivanan (2009) and Saha & Santra (2014).

Molecular characterization

The isolates after morphological identification and purification were molecular characterized by DNA isolation, PCR amplification and sequencing of the 16S rRNA gene. 16S rRNA gene sequences has been useful in phylogenetic studies at the genus level, its use has been questioned in the case of closely related species groups such as Bacillus, where insufficient divergence in 16S rDNA prevented the resolution of strain and species relationships. The sequences were submitted to GenBank and accession numbers were obtained. The 20 accession numbers obtained were MH194570, MH194185, MH194186, MH194187, MH194188, MH194189, MH194190, MH194191, MH194192, MH194193, MH194194, MH194195, MH194196, MH194197, MH194198, MH194199, MH194200, MH194201, MH194202 and MH194203.

Phylogenetic Analysis

A phylogenetic tree was constructed using MEGA 5.10 software using the UPGMA statistical method and Kimura 2-parameter substitution model. Phylogenetic trees explain about the interrelationship of strains in a sample. They are useful and reliable tools of molecular taxonomy. The relative position of an unknown strain in a well-constructed phylogenetic tree can give a lot of information about the probable affinities of the strains and also its evolutionary progenitors. Phylogenetic relationship based on partial 16S rDNA sequences of selected enzyme producers from municipal solid waste and related taxa is represented in Fig. 1. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test with 1000 replications. From the phylogram it was understood that the enzyme producing bacteria isolated from municipal solid waste can be divided into different clades. The most prominent organisms in the tree belong to the genus Bacillus which was grouped into a large clade. *Pseudomonas* and *E.coli* were seen separately in the phylogenetic tree.

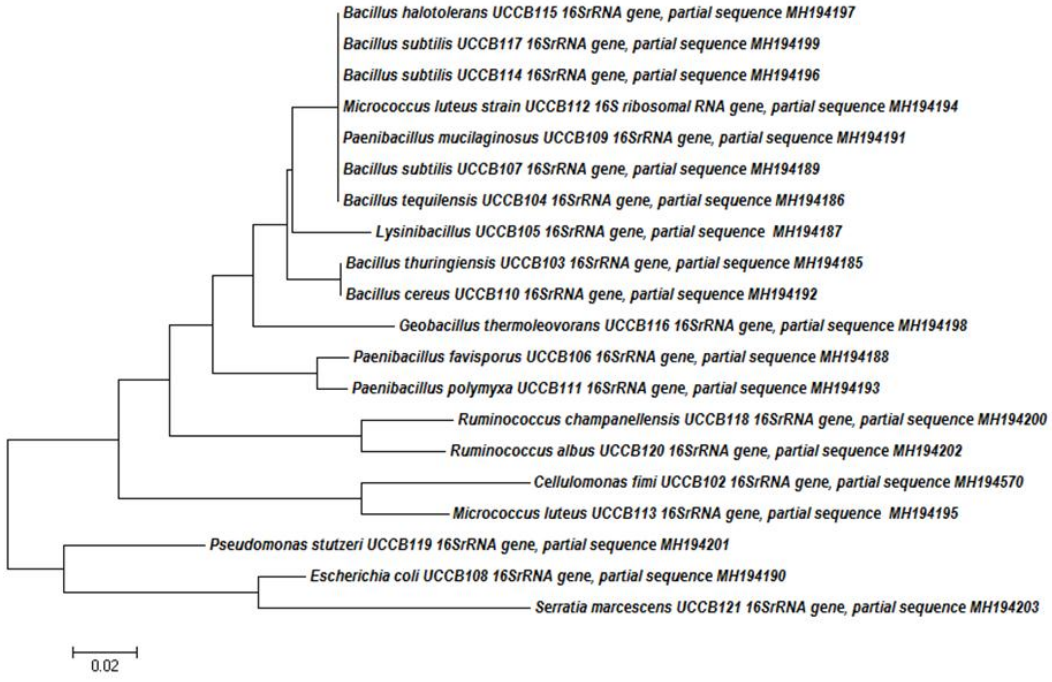

Figure 1: Phylogenetic tree of the based on partial 16S rDNA sequences of 20 enzyme producing bacterial strains isolated from Municipal Solid Waste

Application of isolated bacterial strains

The exploitation of the metabolic versatility of microorganisms is advantageous in biological waste treatment but the actual number of degraders of a target compound in a mixed culture may only represent 5-10% of the microbial community (Sayler et.al.1984). To prepare successful microbial consortium, bacterial cultures must be compatible with each other in order to concomitantly produce all these enzymes required for the degradation of kitchen wastes (Sarkar et.al 2011). These 20 different enzyme producing bacterial isolates were combined with each other by permutation combination in order to make different bacterial consortia. Table 2 shows that 10 different bacterial consortia were prepared of which all consortia showed the best compatibility when gram staining was performed. Degradation of the kitchen wastes in different substrates such as rice husk, rice ash and coir pith were monitored by gradual decrease in the volume for 30 days (Fig. 2,3). The bacterial consortia were used in degrading kitchen waste. Fong & Tan (2000) have also reported the use of bacterial consortia for degradation of organic wastes.

Table 2: Combination of the Selected Bacterial Consortia

Sl.No.	Consortia Composition
1	T3, TT5
2	TT7, M4
3	Wm7, P1
4	T4, P2
5	B3, BR2
6	Bb2, BW2
7	M6, Wm8
8	T2, M5
9	Bw1, Mu12
10	Bb4, L6

Isolation, screening and identification of potential strains that produce industrially important enzymes are prime step in development of any industrial enzyme production. Primary identification is done through gram staining methods. The back bone of bacterial systematic has been derived from the 16S rRNA gene sequence based phylogeny and is the most broad spectrum method used for bacterial classification and identification. 16S rRNA gene sequence analysis is proposed as a species-specific identification tool which can provide fine sensitivity down to single cell detection. 16S rRNA gene sequence analysis has established the major role of relationships among nucleotide sequences in the definition of bacterial species. They are stable molecular markers and are ubiquitous, functionally constant and conserved and are less subjected to lateral gene transfer, and therefore 16S rRNA-based phylogeny of higher taxa is in good agreement with analyses retrieved from genomic approaches. The strains selected for the preparation of bacterial consortium were *Cellulomonas fimi*, *Bacillus thuringiensis*, *Paenibacillus favisporus*, *Lysinibacillus*, *Bacillus tequilenis, Geobacillus thermoleovorans*, *Micrococcus luteus*, *Bacillus subtillis*, *Bacillus halotolerans*, *Escherichia coli*, *Bacillus sp.*, *Paenibacillus polymyxa, Bacillus cereus*, *Paenibacillus mucilaginosus, Ruminococcus champanellens*, *Serratia marcescens*, *Ruminococcus albus* and *Pseudomonas stutzeri*. Bacterial consortia were used for degradation of the solid waste using coir pith, rice husk and rice ash as a substrate. The degradation was faster and with no odour. Thus it proves that they are effective in waste degradation also. The potential strains isolated from different waste dumps showed the production of many industrially important enzymes. These bacterial strains can be isolated for large scale production of enzymes. And also these strains can be modified for easy degradation of waste material or by using the waste material as a substrate for the growth of microbes that are involved in producing bulk quantity of industrially applicable enzymes. The high level of proteolytic activity of the bacterium suggested its suitability in industrial applications and can be exploited commercially in near future.

Figure 2: Addition of waste and bacterial consortia into coir pith, rice husk ash and rice husk

Figure 3: Effective waste degradation by using bacterial consortia

MATERIALS AND METHODS

Collection of samples

Samples were collected from different municipal dumping waste areas of Aluva , Angamali , Kochi. Samples were then brought to the laboratory for microbial study. Samples collected were screened for the presence of amylase degrading, cellulose degrading, protease degrading and pectinase degrading microbes. For this standardised isolation procedures were carried out.

Isolation, identification and maintenance of bacterial culture

Samples collected from different locations were transferred to laboratory. Serial dilution techniques were used for the isolation of bacteria. In this technique sample suspension was prepared by adding soil mixed with waste (1g) was added to 9 ml of sterile water (the stock) and shaken vigorously for at least 1 minute. A homogeneous suspension was obtained. Sterile dilution blanks were marked sequentially starting from stock and10^{-1} to 10^{-7}. One ml from the stock was transferred to the 10^{-1} dilution blank using a fresh sterile pipette. One ml from the10^{-1} dilution was transferred to the 10^{-2} tube for each succeeding step then from the 10^{-2} to the 10^{-3}, then from the 10^{-3} to the 10^{-4}, followed the same processes upto 10^{-7} dilution. From each dilution tube 0.1 ml of dilution fluid was transferred into Nutrient Agar culture media (Beef extract-0.3g, Peptone-0.5g, NaCl-0.5g, Agar- 2g, Distilled water-100ml). After this the inoculated plates were incubated at 37^{o}C for 3-4 days. The microbial strains were then morphologically identified by using gram staining method. The identified microbial strains were then inoculated to nutrient agar slants and stored at low temperature 4^{o}c and also preserved in 20% glycerol vials at -80^{o}C.

Extraction of DNA

For the extraction of DNA, a sample of 2 ml bacterial cell suspension (18 h old bacterial cell suspension grown in Luria–Bertani broth) was centrifuged at 8000 g for 5 min at 4°C. The supernatant was discarded and the cells were resuspended in 400µl of freshly prepared STE buffer(0.1M- NaCl, 0.01M- Tris Cl, 0.001M –EDTA), and centrifuge the tubes for 8000rpm for 2 minutes. The pellets was again resuspended in 200µl of TE buffer (10Mm-TrisCl, 1Mm EDTA). 100µl of Tris-saturated phenol (pH 8.0) was added to these tubes followed by vortex mixing. The tubes were then centrifuged at 13000rpm for 5 minutes at 4^{o}C. 160µl of upper aqueous phase was transferred to a clean microfuge tube. Add 40µl of T buffer to make

up to 200µl and mixed with 100µl of chloroform. The tubes was then centrifuged at 13000rpm for 5 minutes at 4^{o}C. 160µl of aqueous phase was transferred to fresh microfuge tube, add 40µl of TE buffer and 5µl of RNase. The tubes were then incubated at 37^{o}C for 10 minutes. Add 100µl of chloroform, mix well and centrifuge for 5 minutes at 13000 rpm at 4^{o}C. 150µl of aqueous phase was transferred to a fresh microfuge tube. The aqueous phase containing genomic DNA and is stored at -20^{o}C.

PCR amplification of extracted DNA

The molecular characterization was done on the basis of 16S rDNA sequence analysis. The DNA isolated was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA was observed. Fragment of 16S rDNA gene was amplified by PCR (EmeraldAmp GT PCR Master Mix)from the above isolated DNA using universal primers 16S F (GAG TTT GAT CCT GGC TCA) and 16S R (ACG GCT ACC TTG TTA CGA CTT). The amplification profile consisted of initial denaturation at 95°C for 5 min followed by 34 cycles of denaturation at 94°C for 20s, annealing at 58°C for 30s and extension at 68°C for 2 min followed by a final extension at 68°C for 10 min. The PCR product was separated on 1% Agarose gel prepared in 1X TAE buffer and stained with Ethidium bromide.

Sequencing and Analysis

Nucleotide sequencing was performed using ABI 3730xl DNA Analyzer at Scigenom Kochi. Sequenced DNA data were compiled and analyzed. The sequence obtained was first screened for vector regions using 'VecScreen' system accessible from the National Centre for Biotechnology Information (NCBI). After removing the contaminating vector regions the sequences were matched with homologous sequence obtained from the GenBank database using the BLAST algorithm (Altschul *et al.,* 1990) available from the NCBI website (http://www.ncbi.nlm.nih.gov).

Phylogenetic Analysis

Most phylogenetic trees are built from molecular data, DNA or protein sequences. In this study the phylogenetic analysis of the 16S r RNA gene sequence was done by Molecular Evolutionary Genetics Analysis (MEGA 5.10) software and the tree was constructed by using the unweighted Pair Group Method with Arithmetic mean (UPGMA).

Application of enzyme in waste degradation

Consortia Preparation

Consortia was made by inoculating isolates in 10ml nutrient broth test tubes separately at 37^{o}C for 3 days with addition of 0.1% starch, casein and cellulose. Optical density was measured by Elisa Reader at 650nm. 10 different consortia were prepared and incubated overnight at 37^{o}C in 120 rpm. The compatibility of the bacterial strains within the consortia was checked by gram staining. Microbial consortium was prepared by inoculating 20 over night grown bacterial strains in 20ml of nutrient. 24 hour incubated cultures were used for the production of enzyme consortia and its effectiveness in degrading kitchen waste was measured. Rice husk, rice ash and rice powder were used as a substrate for waste degradation.

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