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STUDY ON PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF BACTERIAL SPECIES ISOLATED FROM DECAYED FRUITS

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ABSTRACT

Most of the methods adopted for processing of decayed fruits and degradation of wastes are not economical. In order to achieve 100% resource recovery from wastes, a novel type of waste treatment is to be followed, so that, the recovered byproducts could be used as biofertilizers. The objective of this study is identification of bacteria for studying their phenotypic and genotypic characters using molecular tools and conversion of wastes into biosolids as useful byproducts. Decayed fruit samples were procured and pure culture of bacterial strains were isolated. The isolated strains were subjected for phenotypic analysis of biochemical tests and genotypic gene expressions of 16S rRNA amplification, after which, the bacterial species identified were two strains of *Bacillus subtilis, Serratia marcescens, Arthrobacter nicotianae* and *Corynebacterium singulare.* The antibiogram activity was performed in all the cultures. From this analysis, strains were found to be resistant to most of the antibiotics.

KEY WORDS: **16S rRNA amplification, Antibiogram, Decayed fruits, Genotypic, Phenotypic**

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INTRODUCTION

Organic wastes are produced by a range of industries – agriculture, food processing and beverages industries[1]. Fruit and vegetable wastes that contribute a particular proportion of wastes could be used as a fertilizer as such or in combination of fish wastes, abattoir wastes and waste activated sludge as a co-substrate[2]. Improper storage, negligent handling and damage during transport of fruit and vegetables contribute a sizeable portion for the loss or decay[3]. This lost and damaged portion could be converted into valuable byproducts by subjecting them to appropriate waste treatment methods. However, due to the presence of pathogens, it could not be used as a fertilizer either[4]. Hence, analysis of the microorganisms present in the fruit wastes is essential, based on which the right treatment process is adopted. Microorganisms are known to utilize the soluble sugars and organic compounds present in the fruit and vegetable wastes and convert into valuable biomass. Hence, the fruit wastes serves as a medium for the growth of a variety of microorganisms such as bacteria, fungus and yeasts[5]. Solid state biomethanation of fruits was successful in generation of biogas[3], which is an added advantage. Although most of the microorganisms are capable of degrading the decayed fruits, only Thermophilic bacteria could withstand a higher temperature of 50-70˚C. Thermophilic anaerobic digestion can accelerate hydrolysis and acidogenesis process and allows methanogenesis to occur at neutral pH[6]. A nonpathogenic *Rhizobium* species CWP G34B, grown both in high and low concentration of glucose showed effective degradation of peels of banana, pineapple and orange[7]. A two stage bioreactor used in anaerobic digestion of fruit and vegetable wastes resulted in conversion of over 95% of volatile solids into methane at a production yield of 420 l/kg of volatile solids added[8]. Different outcomes in terms of Saccharification and ethanol production was achieved in a study involving conversion of fruit and vegetable wastes using pure culture fermentation and co-culturing of mesophilic and thermophilic bacteria and yeasts[9]. Currently, there are only a limited number of studies in the analyses of microbiota present in decayed fruits and their morphological and biochemical properties. Although several reports are available about microorganisms of human pathogens, there is very little research in the identification, characterization of bacteria responsible for degradation of fruits as there is not much exploration of their pathogenic study. Hence, the present study is an attempt to identify the bacterial strains from decayed fruit wastes. Bacterial consortia produced specific enzymes (amylase, lipase, protease, cellulose) required for kitchen wastes degradation. Reduction up to 65% of kitchen wastes was achieved in a selected consortium in just 21 days[10].

MATERIALS AND METHODS

Sample Collection

Decayed fruit sample was collected from the corridors of Gandhi market, Tiruchirappalli, India. The collected sample was brought to the lab in a sterile plastic container and stored at 4˚C until use.

Isolation of bacteria

To isolate the bacteria, serial dilution was done in the range of 10^{-1} to 10^{-9}. The dilutions selected from 10^{-4} to 10^{-7} were taken for the study. The serial dilutions were then spread on nutrient agar plates and incubated at 37˚C for 24h. After incubation, size and color of the colonies were observed. Then the strains were purified by repeated streaking of single colonies in nutrient agar plates. Pure bacterial strains obtained were kept in Nutrient agar slant at 4˚C for further use.

Phenotypic characterization of the strain

The strains were tested for different culture characteristics, morphological and biochemical properties such as utilization of different carbohydrates as carbon source, Indole, Methyl red, Voges Proskauer and Citrate utilization test (IMViC), catalase, oxidase, nitrate reduction, urease, Triple Sugar Iron agar test (TSI) etc. by the conventional methods. A single well isolated colony from nutrient broth was used for each of the test and incubated at 37˚C for 24h. After

incubation, the color reactions were read (some with the aid of the added reagents).

Genotypic characterization of the strain

Extraction of genomic DNA from the bacterial isolate was done using the Cetyl Trimethylammonium Bromide (CTAB) protocol contributed by Wilson (1997)[11]. The isolated DNA was subjected to PCR amplification using the specific universal primers: (A8F) Forward: AGAGTTTGATCCTGGCTCAG and (A1492R) Reverse: TACGGCTACCTTGTTACGACTT. The PCR amplified products were run on 1.4% agarose gel electrophoresis. After this, the gel was viewed under gel documentation unit (BIO-RAD Gel Doc™ XR+ with Image Lab™ Software). The PCR amplified product was sent to Xcelris Labs, Ahmedabad for partial 16S rRNA sequencing. The nucleotide sequence was initially analyzed at Blast-n site in NCBI server (http://www.ncbi.nlm.nih.gov./BLAST) and corresponding sequences were downloaded. The sequences were also analyzed using the MEGA – 4 software to construct the phylogenetic tree[12].

Determination of Antibiotic resistance

The antibiotic resistance pattern of the isolated bacteria against different antibiotics was determined by disc diffusion method[13, 14]. The following antibiotics (HiMedia, India) were used. The concentration of the antibiotics used was in µg/disc. The symbols and concentration of respective antibiotics are: Penicillin (10 units), Gentamicin (10 mcg), Erythromycin (15 mcg), Ampicillin (10 mcg), Oxacillin (5 mcg), Tobramycin (10 mcg), Vancomycin (30 mcg), Cefotaxime (30 mcg), Ciprofloxacin (10 mcg), Cefuroxime (30 mcg), Streptomycin (10 mcg) and Chloramphenicol (30 mcg). The plates were incubated at 37°C for 24h. After incubation, their zone of inhibition were measured in mm and interpreted with NCCLS recommended values[15].

RESULTS AND DISCUSSION

The bacterial strains were isolated from the decayed fruit wastes. The results of morphological and biochemical tests performed for the strains 1 to 4 are shown in Table 1.

Table 1
Biochemical Tests Analysis

S. No	Characteristics	Results				
		Strain 1	Strain 2	Strain 3	Strain 4	Strain 5
1	Gram's staining	+	+	+	+	-
2	Shape	Rod	Rod	Rod	Rod	Rod
3	Motility	Motile (Swarming)	Motile (Swarming)	Non motile	Weak motility	Motile
4	Indole production	-	-	-	-	-
5	Methyl red	-	-	-	-	-
6	Voges Proskauer	-	-	+	-	+
7	Triple Sugar Iron Agar test	A/A, No H_2S	A/A, No H_2S	Variable	A/A, gas, No H_2S	K/A, No gas, No H_2S
8	Citrate utilization	-	-	-	+	+
9	Urease	-	-	-	-	-
10	Oxidase	+	+	-	-	-
11	Catalase	+	+	+	+	+
12	Nitrate reduction	+	+	-	+	+
13	Ortho Nitrophenyl beta Galactoside	-	-	Variable	Variable	+
14	Starch hydrolysis	+	+	-	-	-

A/A – Acid slant / Acid butt, H_2S – Hydrogen disulphide,
(+) – Positive, (-) – Negative, K/A – Alkaline slant / Acid butt.

Isolation of DNA and PCR Amplification of 16S rRNA

The genomic DNA was successfully extracted from the bacteria. All strains were confirmed after 16S rRNA gene amplification after viewing under gel document was run on 1.4% agarose gel electrophoresis (Fig.1).

PCR Amplified product

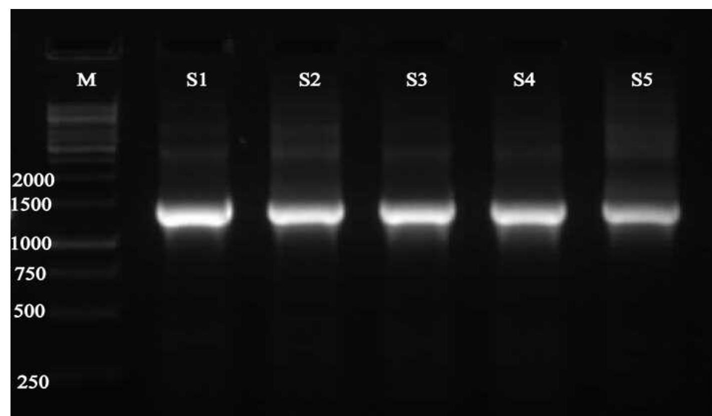

Figure 1
1.4% agarose gel electrophoresis; M – Marker (1 kb ladder); S1 – Strain 1; S2 – Strain 2; S3 – Strain 3; S4 – Strain 4; S5 – Strain 5.

Phylogenic tree analysis

The 16s rRNA sequences of this strain showed 97% of sequencing similarity with *Bacillus methylotrophicus* strain (JF899287) and other Bacillus species groups. Based on the phenotypic and genotypic analysis, the strains were identified are: two strains of *Bacillus subtilis, Corynebacterium singulare, Serratia marcescens* and *Arthrobacter nicotianae.* The sequence of the species was submitted to GenBank and accession numbers were acquired. Accession Numbers: *Bacillus subtilis* strain 1: KF041445 (KTSMBNL-30), Strain 2: KF469298 (KTSMBNL-35); *Arthrobacter* nicotianae – KF431954 (KTSMBNL-34); *Corynebacterium singulare* – KF431953 (KTSMBNL-32); *Serratia marcescens* – KF431952 (KTSMBNL-31). The genetic similarity of four cultures is shown in NJ- tree (Fig. 2).

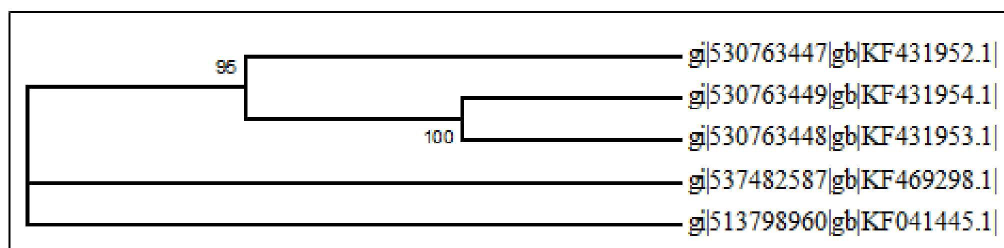

Figure 2
Phylogenetic tree of the isolated bacteria Bacillus subtilis (both strains), Corynebacterium singulare, Arthrobacter nicotianae, Serratia marcescens

Antibiotic Sensitivity Test

From the antibiotic sensitivity tests, zone of inhibition for all the strains was measured in mm after incubation time. The zones of inhibition interpret the resistance (R), intermediate (I) and sensitivity (S) pattern of the strains. The results are shown in the table 2.

Table 2
Antibiotic Sensitivity Analysis; R – Resistant, S – Sensitive, I – Intermediate

Antibiotics	Antibiogram Activity				
	B. subtilis (KTSMBNL-30)	*B. subtilis* (KTSMBNL-35)	*C. singulare* (KTSMBNL-32)	*A. nicotianae* (KTSMBNL-34)	*S. marcescens* (KTSMBNL-31)
Penicillin (10 units)	R	R	R	R	I
Gentamicin (10 mcg)	R	R	S	S	I
Erythromycin (15 mcg)	I	I	S	S	R
Ampicillin (10 mcg)	I	I	R	S	S
Oxacillin (5 mcg)	R	R	R	S	S
Tobramycin (10 mcg)	R	I	S	S	S
Vancomycin (30 mcg)	R	R	R	R	S
Cefotaxime (30 mcg)	S	S	S	I	I
Ciprofloxacin (10 mcg)	S	R	S	S	S
Cefuroxime (30 mcg)	R	R	R	S	S
Streptomycin (10 mcg)	I	I	R	I	S
Chloramphenicol (30 mcg)	S	S	S	S	S

DISCUSSION

The results obtained for the morphological and biochemical analysis of the bacteria isolated from decayed fruit samples indicate that the strains are either mesophilic or thermophilic in nature. All the strains identified were rod shaped. In this study, the 16S rRNA based bacterial community analysis was performed. Based on the antibiotic sensitivity results, it could be assumed that these strains are sensitive to nearly 75% of the antibiotics used. Also, the work suggests the thermophilic digestion of food wastes, stressing the importance of neutral pH and mesophilic and thermophilic temperature range for the efficiency of the process. Other thermophilic bacilli such as *Anoxybacillus flavithermus* and *Geobacillus* spp are found to be bacterial contaminants in the dairy industry and produce highly heat-resistant spores. Though they are non pathogenic, their presence indicates poor hygiene in dairy processing units[16]. In another study, it was reported that application of both ATAD and composted sewage sludge to a peat-based potting mixture increased plant biomass and fruit yield of pepper plants which is attributed to biological properties of the substrate mixture[17]. Kaur and Arora (2012) in a study have identified 4 strains of cellulose degrading bacteria out of 21 strains and standardized the optimum working conditions for biomass utilization[18]. Ragaert *et al.,* (2006), in a study has explained that microbiological processes could play an important role in degradation of strawberries by producing ethanol as a precursor the physiological production of the off-odor ethyl acetate[19]. Mesophilic anaerobic digestion of fruit and vegetable wastes, chicken manure in a continuous stirred tank reactor used for digesting cattle slurry was carried out for production of methane. Increasing the proportion of fruit and vegetable wastes from 20% to 50% improved the methane yield. In a study of degradation of fruit and vegetable wastes, different fruit parts within the same variety showed different yields of methane in orange, pomegranate, grape vine and sapota providing a database on extent and rates of conversion of fruits and vegetable solid wastes[20].

Clostridium straminisolvens and *Clostridium clariflavum* – like organisms were found to be major components in the microbial communities present in cellulolytic consortia enriched with thermophilic biocompost[21]. Culture-independent analysis of bacterial community in bioreactors operated at both thermophilic and mesophilic temperatures revealed that the bacterial community supported by mesophilic reactors supported a greater number of bacterial populations than the thermophilic reactors[22]. A study involving consortium of *Bacillus subtilis* (B1U/1 and D3L/1) and *Pseudomonas* sp (RAT/5) for composting of organic wastes resulted in reduction of C/N ratio, NH_4^+ and NO_3^- ion concentration giving the mature compost a pH of 7.0 ± 0.2 (Pan *et al.,* 2012)[23]. Antibiotic resistance pattern may also be due to geographic variations observed in different strains of Gram negative bacilli (Prabhakar *et al.*)[24]. Mere analyses of phenotypic and

genotypic characters of the identified bacterial strains do not fulfill the resource recovery from decayed fruits. Physico-chemical analysis of biosolids and subjecting it to the appropriate mode of treatment could fetch valuable byproducts besides reducing the toxicity of the feed. One such process is autothermal thermophilic aerobic digestion. It provides a rapid, reliable and easy-to-operate method of treating biosolids. Piterina *et al.,* in a study have examined the thermal inactivation of pathogens in full scale ATAD system resulting in pathogen reduction and their absence at the post-treatment stage[25].

CONCLUSION

Our study has thrown light on the presence of diverse group of organisms in the decayed fruits samples which when analyzed, exhibited distinguishing colony morphology and biochemical characteristics. Phenotypic and genotypic characterization was carried out only for a single strain i.e., *Bacillus subtilis*; analysis of other strains are yet to be carried out. However, study of pathogenicity of these strains is the need of the hour so that the decayed fruit samples could be treated before using as fertilizer. With the increasing demand for a suitable and appropriate process for treatment of fruit and vegetable wastes other than aerobic and anaerobic, autothermal thermophilic aerobic digestion could serve as an alternate method, thanks for the advantages including less time consuming, valuable byproducts and cost-efficiency.

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