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Synthesis of a Novel Microbial Consortium useful in Treatment of Synthetic Polymers & Municipal Solid Wastes and its Effect on the Plant Growth and Environmental Engineering

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Abstract

Scientists have recently discovered that microbial action is enhanced when they are used in form of Consortia than as their individual species. Bio-organic wastes such as human wastes and municipal solid wastes contain millions of tons of fertilizer equivalents, which is roughly 20% to 30% of global industrial fertilizer production, annually. To maintain agricultural yields at high levels over the years, the nutrients removed by crops have to be replaced. Current research work focuses on developing a sustainable technology to treat bio-organic waste such as human excrements into agriculturally useful organic manure. This study also aims at exploiting the power of natural and unmodified microbial flora as consortia for degrading human manure as well as synthetic polymers such as PVC, PU, Nylon etc. Our work shows the novel enzymatic role of microbial consortia and suggests their use in breakdown of wastes and polymers while increasing nutrient quality of organic wastes.

Keywords: *Microbial consortia, Human Manure, Khjedhal method, Synthetic Polymers*

Introduction

With the increasing urbanization, there is a steep rise in consumption of natural resources and it is resulting in accumulation of Municipal Solid Waste (MSW). Sustainable use of natural resources and reuse of wastes has become an important global concern which helps to promote the efficiency of ecological system and also human health by decreasing disease transmission and can contribute to an increase in agricultural yield. In underdeveloped and developing countries, malnutrition constitutes approximately 14% of global burden of disease, which supersedes the sanitation related disease, which is only 3.4% (Lopez *et al.*, 2006). Human wastes and municipal solid wastes contain millions of tons of fertilizer equivalents, which is roughly 20% to 30% of global industrial fertilizer production, annually (Winker *et al.*, 2009). To maintain agricultural yields at high levels over the years, nutrients removed by crops have to be replaced. For example, urine is rich in nitrogen, which is most limiting nutrient for plant growth while feces are rich in phosphorous, potassium and recalcitrant organic matter which can give substantial yield, especially on poor soil (Jonsson*et al.,* 2004). This data reflects the urgent need of sustainable technologies of developing agricultural practices towards improving the nutrient quality of foods without compromising on the safety of environment. Hence, the developing nations like India are in great need of sustainable reuse technologies, which not only prevent disease transmission but also can promote agriculture. Hence, the current research focuses on developing sustainable technologies to treat microbial based conversion of bio-organic waste such as human excrements into agriculturally useful organic manure. Another important problem which has emerged with increased industrialization & technology is uncontrolled use of synthetic polymers or plastics.

Most of the industrial and technological revolutions of the 19th and 20th centuries may be attributed to the development of Plastics. During the past 30 years plastic materials have been used extensively in clothing, shelter, transportation, construction, medical and leisure industries because they are lightweight, low cost, extremely durable and relatively unbreakable. A very general estimate of worldwide plastic waste generation is annually about 57million tons. Other examples include polyesters used in clothing, polystyrene used in insulation, and silicones used in health, Nylon used in

clothing and other products. Natural polymers serve as source of energy and physical strength for organisms and they are the part of metabolism of organisms. In contrast, artificial polymers are useful due to their strength and adaptability. In general, degradation of any polymer follows a sequence in which the polymer is first converted in to its monomers, after which the monomers are further mineralized. Most polymers are too large to pass through membranes of cells, so they must first be depolymerized to small monomers only after that they can be absorbed and degraded through the action of digestive enzymes within microbial cells such as bacteria and fungi. Soil microbes can initiate the degradation or de-polymerization of many natural polymers such as starch, cellulose, and hemicellulose through the action of secretary enzymes, which are released into the soil and these enzymes then begin the breakdown of the polymers. In addition, microbial exudates (other than enzymes) can create a typical environment in which natural polymers become chemically unstable through mechanisms such as oxidation. Polymers are degraded in a particular sequence through abiotic and biological reactions and the standard laboratory tests for measuring such biodegradation may not be able to detect the changes which happen to a polymer. The rate of degradation depends on the specific environmental conditions and nature and type of microorganisms present in that environment. Polymers such as plastics do not easily break down by the microorganisms of the natural environment because of their excessive molecular mass, high number of aromatic rings, unusual bonds or halogen substitutions and other chemical structures. Because of this, plastics remain in the environment for a very long time without any severe damage and the large scale accumulation of unused plastics in the biosphere results in severe environmental pollution through the disturbances in the ecological balance. These problems have made plastic waste a major focus in the management of the solid waste. Microorganisms have differential choice for the degradation of polymers, which may depend both on the chemical structure of a polymer and the kind of enzymes released by the microorganisms and their mutual interaction. Microorganisms secrete a variety of enzymes into the soil environment or where they dwell, which begin breaking down of the polymers present in the vicinity. These enzymes broadly are of two types: i) intracellular enzymes (Endo) and ii) extracellular enzymes (exozymes). The latter are secreted outside of the microorganism in to their environment.

The present study specifically aims at exploiting the power of natural and unmodified microbial flora of soil and aquatic systems in form of their consortia for degradation or deterioration of both, bio-organic derivatives of human waste as well as synthetic polymers such as PVC, Poly Ethylene, PU & Nylon. Being first of its kind of attempts, our results shed a light on previously not well known enzymatic roles of microbial consortia and results in increased nutrient quality of organic wastes.

Materials and Methods

Media Preparation & Cultivation of Microorganisms

Routine bacterial culture media such as Nutrient Agar, Nutrient Broth, Luria Broth, Luria-Bertani Agar (LB Agar) were prepared as per manufacturer (Hi Media) instructions. For cultivation of fungi, YPD broth or YPD agar media (1% Yeast Extract, 2% Peptone, 2% Dextrose & 1.5% Agar (HiMedia)) were used. Selection media such as MacConkey or EMB agar plates, Mannitol Salt Agar plates were prepared as per Manufacturer's (HiMedia) instructions. Bacteria were cultivated at 37^{O}C while fungi were grown between 18 to 42^{O}C depending on strain type. All procedures of bacterial culturing performed as previously described. (VKN *et al*., 2014).

Media optimization procedures

Modified Czapek-mineral salt broth per litre supplemented with 0.5% starch, tributyrin and milk powder each was used. 1% of each successfully compatible consortium was inoculated separately in 250 ml of specialized media and incubated at 37°C in 120 rpm till 5 days. After every 24 hours, 5 ml of each consortium was taken out to check the production of amylase, protease, lipase and cellulase that are responsible for the degradation of bio-organic wastes. For checking the polymer degradation, Synthetic Dextrose Agar (BD biosciences) was used by mixing the media with 0.02µM sized Poly Vinyl Chloride (PVC), Poly Urethane (PU), Low Density Poly Ethylene (LDPE) and Nylon were taken in different culture media.

Enzymatic characterization of Microbial species

The exo-zyme activity of microbial species was determined using biochemical reactions. Enzymatic assays were performed by centrifuging the microbial consortia at 15,000 rpm for 10 min. The supernatant was used for enzymatic assay. The experiments were carried out in duplicates and standard error was calculated.

Lipase assay: Lipase activity was assayed titrimetrically at pH 8.0 with a standard tributyrin as substrate as described earlier.1ml tributyrin was mixed with 3ml of Tris HCl (pH 8.0) to form emulsion. 1 ml of the enzyme was added to the emulsion. The mixture was incubated at 50°C for 30 min. The released fatty acids were titrated with 50mM NaOH. One unit of activity was defined as the amount of enzyme which liberated 1µM butyric acid per min under standard conditions.

Protease assay: The enzyme extract suitably diluted, was mixed with 50mM glycine - NaOH buffer (pH 9) to make 1 ml volume. 1ml of 1% casein (substrate) was added and incubated for 10 min at 60°C. The reaction was stopped by addition of 0.5 ml TCA (20%, w/v). The mixture was allowed to stand at room temperature for 30 min and filtered. 1 ml of the filtrate was mixed with 5 ml

of 0.5M Na2CO3 solution. 0.5 ml of Folin & Ciocalteu's (phenol reagent) reagent was added and kept in dark to develop the blue color. It was estimated spectrophotometrically at 660nm against tyrosine as standard. One unit of protease activity was defined as the amount of enzyme required to liberate1g tyrosine per milliliter in 1 min under the experimental conditions used.

Amylase assay (DNSA 3, 5 dinitro salicylic acid methods): One ml of 1% starch was incubated with different dilutions of the enzyme extract and 1ml of citrate-phosphate buffer (pH 6.0) and was incubated at 50°C for 30 min. The reaction was stopped by adding 2 ml of DNS and kept in boiling water bath for 10 min and absorbance was recorded at 540nm against glucose as the standard. One unit of enzyme activity is defined as the amount of enzyme, which releases 1µmole of reducing sugar as glucose per minute, under the assay conditions (U/ml/min).

Determination of Waste Degradation

The consortia capable of producing all these enzymes concomitantly were further selected for laboratory trials. Laboratory trials were carried out in 5 kg samples collected from Sulabh International twin pit toilet. Each sample was inoculated with 5% of consortium by evenly mixing the inoculum with the wastes and kept under natural condition for 3 months with 15 days of interval to observe the visual rate of degradation.

Isolation & staining of Soil borne fungi & microbes

Various microorganisms were isolated from wet soil by mixing soil with Starch Casein Agar medium and Mineral Salts Agar medium at a concentration of 2% (w/v) and plates were incubated at 37^{o}C to 42^{o}C for 7-10 days depending upon growth of the strain. Microbes were studied by Gram staining procedure and photographed under 1000X magnification using oil-immersion microscopy (Olympus). Specific microbes such as Bacillus cereus, Streptomyces, Pseudomonas, Actinomycetes, *Aspergillus niger* & *Sacharomyces cerevisiae* are some of the bacterial and fungal species which are isolated or procured from MTCC. The pure cultures of these microbes along with soil borne microbes were used for preparation of microbial consortia.

Enumeration of Anaerobic microbes

To cultivate and enumerate anaerobic microorganisms, a candle jar method has been employed in which microbes under investigation were plated on selection media and incubated by using Candle Jar method (Dubey&Maheswari, 2010). These cells were enumerated after 24 to 48 hr incubation directly under colony counter.

Constitution of microbial consortium

Microbial consortium has been prepared aseptically by taking log phase grown bacterial & fungal cells (10^{6}cells/ml), by determing their O.D at 595nm (Sambrook *et al*., 2000). Cells were collected by centrifuging at 5000rpm for 10 mins and briefly washed using sterile Phosphate Buffer Saline (PBS). This cell pellet was added to a 10% diluted molasses solution (Bengal chemicals) and thoroughly mixed. The solution has been further diluted (10:100) with double distilled autoclaved water and the molasses-cell solution was incubated at 37^{o}C for one week while periodicallymixing the contents.

Application of microbial consortium on human waste & polymer waste

The microbial consortium made has been applied on human derived waste collected aseptically from Sulabh International, Gurgaon at 2-5% concentration (w/v) and incubated at 30 to 37^{o}C and observed for different time periods. A time point study was performed by taking samples for analysis of total dry wastes present, against the untreated control setup. For experiments on synthetic polymers, different polymers such as Poly Ethylene, PVC, Poly Urethane & Nylon were purchased from Sigma Aldrich. Microbial consortium or groups of microbial species were added to each of the polymer in Nutrient broth solutions at 5% concentration (w/v). These polymer samples along with the microbial cultures were incubated at 37 & 42^{o}C, for 3 to six months, while replacing the culture after every 15 days of interval. Polymer samples were collected for analysis at regular intervals.

Electron Microscopy and Image Analysis

The treated and untreated sample of PVC and Nylon 6 were tested under SEM to check the surface phenomena at Advanced Instrumentation Research Facility of Jawaharlal Nehru University (JNU), New Delhi. Specimens were cut according to dimension of stabs and they were carefully placed on the carbon tape so that it sticks to the stabs perfectly. The specimen was treated for gold coating. After gold coating the stabs are placed in the chamber present in the SEM machine. The surface is observed at different magnification and at different area using a joystick. The image is seen on the monitor and photo was captured.

Application of microbial consortium on duck weed

Microbial consortium at 2% was added to 1gm of 20 days old duck weed, aseptically and microscopy of roots, at 400x magnification was performed before and after addition.

Estimation of total Nitrogen by Kjeldahl method

The total Nitrogen present in the microbial consortium has been estimated by Kjeldhal method as described by Manivaskam, 1996.

Estimation of Potassium content

To estimate the potassium content in garden soil, microbial filtrate and toilet derived manure, we have used

the Flame photometer (Labtroics, LT671), using standard operational procedure. Distilled water was taken as control.

Tomato Plant study

It was essential to take tomato plants of same age, variety and have similar or identical genetic constitution. For this, tomato plants were raised starting from seeds and plant seedlings were grown. Among them, 15 Tomato Plants of equal height and morphology were selected. They were made in to three groups of five plants in each group. One group (A) of tomato plants was transferred to pots containing sterilized soil, which was achieved using autoclaving. One group (B) of plants was used for supplying with raw water. Another group (C) was used for testing the effect of Microbial consortium on tomato plants. Only one month old tomato plants were considered for experimentation. Group A plants were given only filtered/distilled water, whereas Group B were given raw water and Group C were added with Microbial consortium at 5% (V/W) of soil weight of pots. Plants were added with respective solutions on regular basis. Changes in their morphology was monitored with an interval of 5 days and various parameters such as number of leafs, plant height, onset of flowering, number of fruits and fruit size was monitored. The comparison was made using Microsoft excel software and other statistical tools

Results & Discussion

1. Microbial Consortium contains both aerobic and anaerobic bacteria

The microbial consortium prepared for the purpose of efficient disposal of human wastes contain microbes such as *Bacillus cereus*, Streptomyces, Pseudomonas, Actinomycetes, *Aspergillus niger*, Rhizopus & *S. cerevisiae* are some of the bacterial and fungal species inoculated. Bacterial cultivation and enumeration under aerobic and anaerobic conditions suggested the presence of these cells at an approximate $1.6x10^7$ and $8.5x10^6$cells/ml, respectively. Further, Gram staining has been performed to understand the nature of prevalent bacterial species, which indicated the presence of several Gram positive microbes and few Gram negative bacterial species. The presence of diverse spectrum of microbes suggests a role in digestion of various complex macromolecular biochemical compounds present in human derived wastes and metabolites. However, the specific nature of the action of these microbes acting on biochemical compounds is under investigation.

2. Human wastes are digested efficiently in presence of microbial consortium

The digestion efficiency of microbial consortium has been evaluated *in-vitro* by adding it to human wastes which includes two pit derived solids and mixture of other bioorganic matter. The microbial consortium mixed wastes were incubated at variable temperatures for different time points as described in material and methods. A 30 day time point study under partial anaerobic conditions revealed digestion of an approximate 70-80% of solids against the control solids where 40-60% of solids were subjected to digestion. It was also conspicuous that the unadditized control sample has developed a green to brown lawn, indicating growth of unwanted nonspecific microorganisms. The experimental setup having microbial consortium has reduced solid contents and remained free of secondary contaminants.

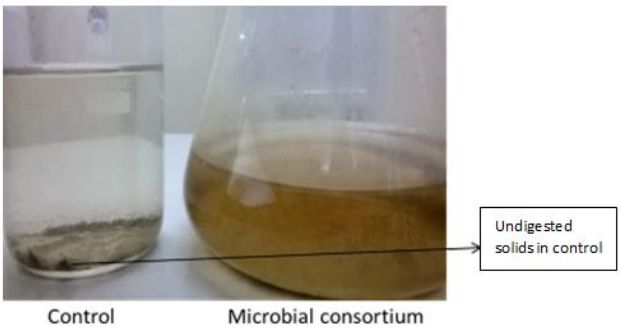


Fig.1: Solids were incubated with & without microbial consortium for 1-3 months. This picture presents the end of digestion period

3. The microbial consortium is rich in total Nitrogen content

The observed promotion of plant root growth raised a possible presence of rich nitrogen content in the microbial consortium. It was known that microbes such as Rhizobium, Nitrobacter and Nitrosomonas can efficiently fix atmospheric nitrogen. The second source of Nitrogen is molasses, which carries trace amounts of nitrogen. Taken together, the promoted plant growth might be directly or indirectly related to presence of nitrogen in the microbial consortium. Hence, the nitrogen content of microbial consortium has been estimated by Kjeldahl method and found to be 1606mg/L, which is a reasonably high content of nitrogen, which might have promoted the plant root growth.

4. A comparative analysis of Potassium (K) content in microbial filtrate & garden soil

It is important to check the Potassium content in garden soil and microbial filtrate as it has an important role in plant growth and metabolism. The Potassium content was found as microbial filtrate: 94.8 mg/L of Potassium against Garden soil filtrate: 10.1mg/L of K.

5. Changes observed in polymer properties in presence of microbes

Various synthetic polymer materials such as poly vinyl chloride (PVC), poly ethylene (PE), High Density Poly

Ethylene (HD-PE), Low Density Poly Ethylene (LD-PE), Poly Ethylene Tetraphthalate (PET), Poly Vinyl Alcohol (PVA), Nylon 6.0 were used for preliminary analysis. Where necessary, powder form of polymer was subjected to ultra- sonication and homogenization to disperse into the solutions. Before microbial treatment, polymers of same size and characters were studied from their mass, molecular weight determination and surface structures were microscopically observed at 1000X magnification. Molecular Weight (MW) of polymers has been determined by dissolving polymers in suitable solvent and measured using Reflective Index based Molecular Weight estimation method or Gel Permeation Chrmatography (GPC). At this point of time we have used number average method of estimating MW. Microbial strains were analyzed from their colony appearance, cell shape and response to staining procedures such as Gram's staining technique.

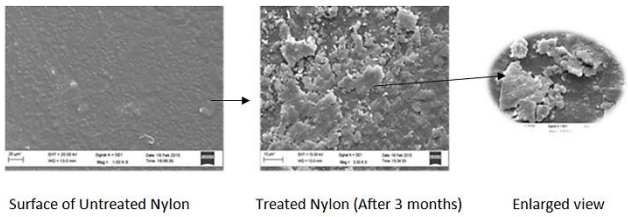


Fig2. Chlorophyll formation in duckweed plant incubated with & without microbial consortium

4. Microbial consortium has a possible role in promotion of plant growth

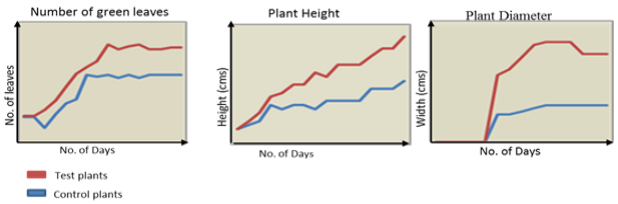
Common observation that pit sites contain good vegetation prompted us to check the interaction of microbes with plant roots and their effect on plant growth. To verify the same,a tiny flowering plant, duckweed was taken and incubated with microbial consortium for 15 or 30 days in the presence of sunlight with proper aeration. A control experiment has been set up with distilled water. Microscopy of duckweed roots of both the experimental setups indicated presence of lush green portion and lengthy roots in microbial consortium treated plants than untreated plants. The observed phenomenon could have resulted from the interaction of microbes with the root of the plant and promoted the metabolic processes required for their growth.

6. Effect of microbial consortium vs. garden soil filtrate on tomato plant growth

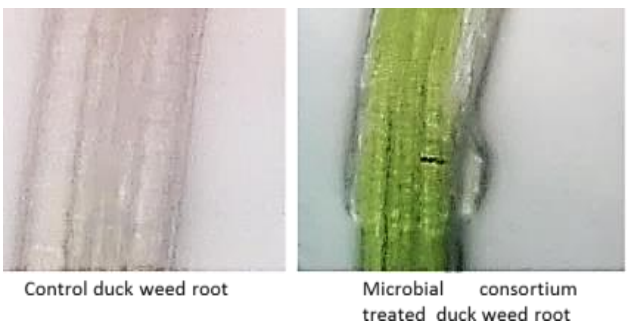
To understand the effect of garden soil and microbial consortium on plant growth, we added garden soil filtrate and microbial consortium to tomato plant seedlings sowed in sterile soil. After 5 to 7 days it was observed that microbial consortium was able to provide habitat for other soil microorganism while supporting the plant growth. The garden soil filtrate also had a positive effect on growth enhancement of tomato plants. However the effect was over shadowed by the effect of microbial consortium. As shown in the photographs there are three pots: test1, test2 and control. We added 50ml of Microbial consortium or garden soil filtrate in each pot and repeated the process with an interval of 6 days, excluding the control setup having sterile soil. It was clearly observed that, the average height of test plants added with Microbial consortium was higher than garden soil filtrate.

Tomato plants under study before fruit harvest (Control, Test-1, Test-2 are arrow marked)

Fig3 A. Tomato plant growth differences between control (without consortium), Test-1 (with consortium & Test-2 (Soil filtrate + Consortium)



B. Graphical pettern depicting differences in growth pattern between Control & Test-1& 2



C. Mini-field scale trial of consortium on tomato plant growth enhancement

Conclusion

With the rapid growth of human settlements and industrialization, the handling of waste generated has become an impossible task with the current technologies available. With the objective of addressing this problem, we have devised a microbial based disposal method for rapid digestion of solid wastes. The system involves selection and enrichment of simple and soil borne microbes, which otherwise are extensively present in soil environment. These microbes produce enzymes which can use varieties of biochemical compounds as their substrates. A plethora of microorganisms are available which perform such function. In our knowledge little or no microbial system has efficiently answered the question in a sustainable manner. A group of microbes such as Actinomycetes, Streptococcus, Pseudomonas and certain subspecies of Bacillus which can co-exist have been brought together and are maintained in a cheaply available molasses like medium. The use of a diluted molasses like solution helped us to minimize the cost and nourished microbes present apart from providing minerals, vitamins and nutrients required for microbial survival. This methodology when fully developed would facilitate the end user to easily follow the three R principle of bioremediation: “Reduce, Reuse and Recycle”. Taken together, our research work helped to create a microbial system with an enhanced fertilizer value which has a potential role in efficient treatment of human wastes and paves a way for improving the agriculture production through increased nitrogen content required for plant growth.

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