Biochemical and molecular characterisation bacterial Isolates from the waste products of Nagaon Paper Mill

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Abstract

Nature has a unique way of degrading the unwanted wastes which are a burden to the society. For this, nature involves the microorganisms, which degrade the waste for their food and in the process gives out metabolites like enzymes such as cellulases, xylanasses, mannoses etc. and antibiotics. So, it becomes a necessity and very interesting to study those microbes for a better developed and cleaner society. Hence, a study has been done on degraded bamboo dust from Nagaon Paper Mill Guwahati, Assam, which is an unexplored area regarding microbial diversity especially on bamboo waste. Six bacteria were isolated from the sample by serial dilution and spread-plate technique. All the strains were gram-positive and spore-forming. With the help of biochemical characterisation, the exoenzyme and endoenzyme activities of the cells were observed. The exo-enzyme activities were studied by tests like hydrolysis of starch, lipid, casein, gelatine etc. Endoenzyme activities were understood by tests like carbohydrate fermentation, H_2S production, catalase, urease, oxidase, IMViC test, nitrate reduction etc. The results of biochemical tests were put in an online tool, the ABIS online tool, accessed through www. tgw.1916.net. Bacterial characterisation and identification depicted that all the isolates belonged to the Genus Bacillus. Identification of the strains was further validated by using 16 s rRNA sequencing.

Keywords: Biochemical characterisation, molecular characterisation, bacterial isolates, bamboo dust

1. INTRODUCTION

Microorganisms are known for their diversity, some are motile and some cannot move, some are photosynthetic and some are heterotrophic. North east India is a biodiversity hotspot, and is known for its rich diversity of flora and fauna; however the region's vibrant microbial diversity has remained largely obscure. Microorganisms are the major organisms responsible for controlling the amount of nutrient cycling and for controlling the amount of nutrient available to plants (Herbot and Robertson 1994).

Microorganisms carry out various biochemical processes to degrade waste materials. This process may be aerobic or anaerobic. Bacteria are the most abundant group in the microbial population of soils (Alexander, 1961) and also the most important microbe for decomposing waste. They 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 (Olson *et al*., 2000; Lynch 1984). In the due course of these decomposition bacteria produces an essential metabolite of great economic value, known as enzyme.

Hence it is essential to explore, preserve, conserve and utilize the unique microbial flora fulfilling emerging needs of society, industries and clean environment. Correct identification of bacterial strains is beneficial for researchers and physicians in many aspects especially for their control or treatment (Hasibe and Dilek, 2011).

When an unknown bacterium is isolated in the laboratory in the pure form, it is usually identified by a combination of information from microscopic observations, i.e., morphology and arrangements of cells: cultural (growth) characteristics on agar and in broth: the gram stain and other staining reactions, the presence or absence of motility: and biochemical tests (tests used for the identification of bacterial species based on the differences in the biochemical activities of different bacteria). Biochemical reactions that occur both outside and inside the cell are precisely controlled by enzymes which can be either exoenzymes (extracellular) or endoenzymes (intracellular). In the laboratory, presence of an exoenzyme is assayed by looking for a change in the substrate outside a microbial cell. (Aneja, 2014)

Endoenzymes are utilized by the cell for further metabolic degradation of carbohydrates and are mainly responsible for synthesis of new protoplasmic requirements and production of cellular energy from assimilated materials. These enzymes function inside the cell. Examples include carbohydrate fermentation, hydrogen sulphide production, nitrate reduction, catalase reactions, urease test, oxidase test, IMViC test. A series of biochemical tests can provide a microbial "fingerprint". (Aneja 2014)

In the absence of molecular data, morphological features combined with certain biochemical tests are performed as the part of identification procedure. In this way, sufficient data is obtained to suggest the identity of unknown bacteria (Ali and Naseem, 2012). Biochemical tests may give an idea of the species niche in the ecosystem. For example, a bacterium that can fix nitrogen gas or oxidize elemental sulphur will provide important nutrients for plants and animals.

STUDY AREA:

The study area of this project is Nagaon Paper Mill, situated on the Guwahati-Nagaon Road, (National Highway No 37) in the Morigaon district of Assam which is the totally unexplored area regarding microbial diversity till date. The geographic coordinates of study area is Latitude 26º 7' 23" N Longitude $92^0$13'24" E. It was established in 1970 as a unit of Hindustan Paper Corporation Ltd and is the first paper mill in the world to produce Kraftpulp in Kamyr Continuous Digester with 100% bamboo as raw material.

Fig. 1.1 Map showing the location of Nagaon Paper mill

Fig. 1.2 Sites of Nagaon Paper Mill

2. Materials and Methods

Sample collection

The sampling area was the degrade bamboo waste corner from Nagaon paper Mill. The degraded bamboo waste (which was our sample) was placed in sterile polythene bag and stored in a refrigerator at 4^0C till use.

Fig.2.1: Nagaon Paper Mill (Sampling site)

Isolation of bacterial strains

Serial dilution and spread plate technique: (Aneja, 2014; Cappuccino and Sherman, 2004)

1g of the bamboo waste was suspended in 9 ml of sterile water to make a microbial suspension. Serial dilutions (up to 10^{-5}) were made by pipetting 1 ml aliquot into 9 ml sterile water. 1 ml aliquot of 10^{-5} dilution was transferred aseptically to sterile petriplate containing sterile, cool molten (45^0C) Nutrient agar and then spreaded using a spreader. The plates were incubated in an inverted position at 37^0C for 24 to 48 hours. Six colonies were picked and then sub cultured in nutrient broth for purification, and labelled as 1, 2, 3, 4, 5 and 6.

- **Identification of bacterial species:** (Aneja, 2014; Cappuccino and Sherman, 2004)

Identification of bacterial species was done by recording the various biochemical activities of the bacterial strains. The different biochemical tests with the bacterial culture by inoculating it into different media, slants and broth as given below:

Tests	Steps	Expected Outcome
Gram Staining	Smears were prepared, heat fixed, then treated with crystal violet for 30 seconds, washed with distilled water, covered smears with Gram's iodine for 60 secs, washed with 95% ethyl alcohol, washed with water, applied safranin for 30 seconds, washed and blot dried.	Bacteria that appear purple are gram-positive; those appearing pink are gram-negative.
Endospore Staining	Smears were prepared, heat fixed, smears were flooded with malachite green, heated the slides to steaming for 5 mins, washed in running water, counterstained with safranin for 30 sec, washed smears in distilled water, blot dried	Endospores appear green in colour
Carbohydrate fermentation(glucose, mannitol, lactose, sucrose)	Sterilized fermentation broth was inoculated with test organisms and incubated at 35 ˚C for 24-48 hrs	Media colour changes from red to yellow
Starch hydrolysis	Sterilized starch agar media was inoculated with test organisms at 35 ˚C for 24-48 hrs	A clear zone surrounding the bacterial growth
Gelatin hydrolysis	Sterilized gelatin agar medium was inoculated with test organisms and incubated at 37˚C for 4-7 days; refrigerated at 4 ˚C for 15 mins	Liquefied cultures following refrigeration
Casein hydrolysis	Sterilized skim milk agar medium was inoculated with test organism and incubated at 37˚ C for 24-48 hrs	Clear area surrounding the bacterial growth
Lipid hydrolysis	Sterilized tributylin agar medium was inoculated with test organisms at 37 ˚C for 24-48 hrs	Clear zone surrounding the bacterial growth
Hydrogen Sulphide Production	Sterilized sulfide indole motility agar was inoculated with test organisms at 35-36 ˚C for 48 hrs	Occurrence of black precipitate
Indole production	Sterilized 1% tryptone broth was inoculated with test organism at 35 ˚C for 48 hrs	Development of cherry red colour in the top of the tube.
Methyl red test	Sterilized MR-VP broth was inoculated with test organism at 35 ˚C for 48 hrs; 5 drops methyl red indicator to each tube	Presence of red colour.

Voges- Proskauer(VP) test	Sterilized MR-VP medium was inoculated with test organisms and incubated at 35˚ C for 48 hrs, added 12 drops of VP reagent I and 2-3 drops of VP reagent II and shaken well	Development of deep rose(pink) colour
Citrate utilization test	Sterilized Simmon's Citrate Agar was inoculated with test organisms and incubated at 37 ˚C for 48 hrs	Change of media colour from green to blue
Urease test	Sterilized urea medium was inoculated with test organisms and incubated at 37˚C for 24-48 hrs	Change of media colour from yellow to red or pink
Nitrate reduction test	Sterilized trypticase nitrate broth was inoculated with test organisms and incubated at 37 ˚C for 24-48 hours;5 drops of sulfanilic acid and alpha naphthylamine added	Change of media colour to red
Catalase test	Sterilized trypticase soy agar media was inoculated with test organisms and incubated at 35 ˚C for 24-48 hrs, allowed 3-4 drops of hydrogen peroxide to flow over the growth	Bubbles of free oxygen gas
Oxidase test	Sterilized trypticase soy agar media was inoculated with test organisms at 37 ˚C for 24-48 hrs; colonies are picked and smeared on oxidase disc	Development of pink colour

Identification was done using the ABIS online tool. It is a laboratory tool for bacterial identification which is open for public use and can be accessed through www.tgw1916. net. It gives result based on the results of the biochemical tests performed.

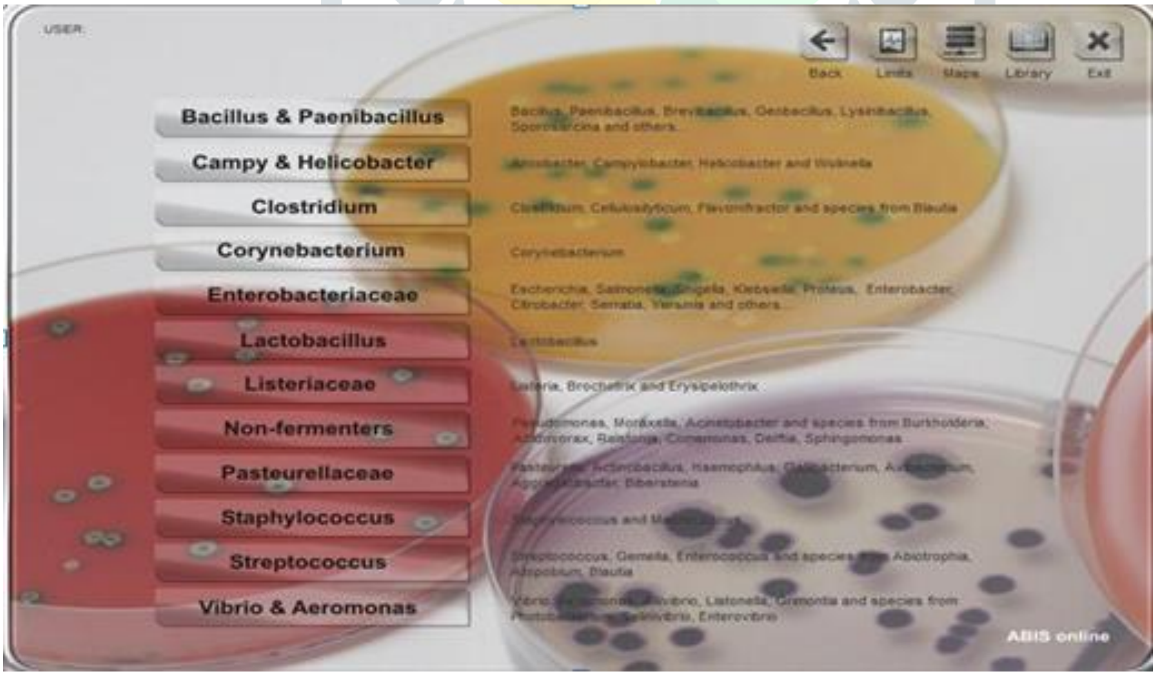


Fig. 2.2: ABIS online identification tool

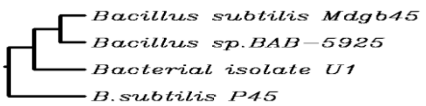
Molecular identification

Using BLASTn, the sequences bearing the highest similarity score with the sequences of the isolates (obtained by 16 s rRNA sequencing) were selected and multiple sequences aligned using Clustal Omega Tool.

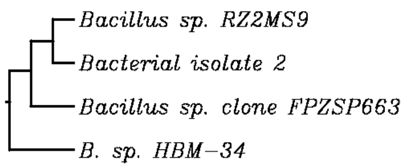
3. RESULTS

Table 3.1: Showing biochemical tests results

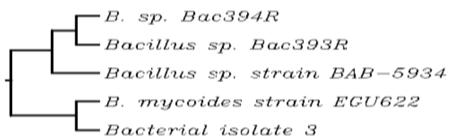
Organism	Gram Stain	Caesin hydrolysis	Gelatin liquefication	Starch Hydrolysis	Lipid Hydrolysis	Lactose	Dextrose	Sucrose	Mannitol	H_2S production	NO_3 production	Indole production	MR reaction	VP reaction	Citrate use	Urease activity	Catalase activity	Oxidase test	Expected organism
1	+	+	+	–	+	–	-	+	-	–	+	–	–	+	+	–	+	+	*Bacillus subtilis*
2	+	+	+	–	+	–	+	–	-	–	+	–	–	+	+	–	+	-	*Bacillus cereus*
3	+	+	+	–	+	–	+	–	+	–	+	–	+	+	+	–	+	-	*Bacillus mycoides*
4	+	+	+	–	+	–	+	–	+	–	–	–	–	+	+	–	+	–	*Bacillus thuringenesis*
5	+	+	+	–	+	–	–	+	–	–	–	–	–	–	+	–	+	+	*Brevibacillus parabrevis*
6	+	+	+	–	+	–	–	+	–	–	+	–	–	–	+	–	+	+	*Lysinibaclus fusiformis*



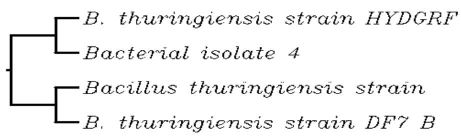
Phylogenetic tree view of bacterial isolate 1



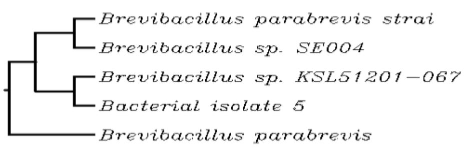
Phylogenetic tree view of bacterial isolate 2



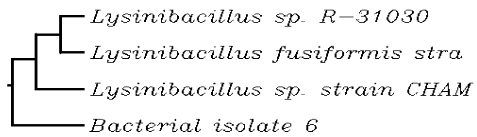
Phylogenetic tree view of bacterial isolate 3



Phylogenetic tree view of bacterial isolate 4



Phylogenetic tree view of bacterial isolate 5



Phylogenetic tree view of bacterial isolate 6

Table 3.2: Showing phylogenetic treeview results

4. Discussion

Works of various scientists like Amin *et al.* (2015) suggested that gram positive and rod shaped bacteria may belong to phylum Firmocutes which includes genera such as *Bacillus*, *Listeria* and *Clostridium*. *Bacillus spp.* and *Clostridium spp.* adapt easily to diverse habitats because of their spores (Parvathi, 2009), while endospore formation is absent in genus *Listeria spp*. *Bacillus spp.* forms oval shaped endospores but *Clostridium spp.* has unique bottle shaped endospores. Another distinguishing feature between Genus *Bacillus* and Genus *Clostridium* is that *Bacillus spp.* secretes catalase to destroy toxic by-products of oxygen metabolism while *Clostridium spp.* does not form enzyme catalase. Thus, *Bacillus spp.* will give positive results in catalase test while *Clostridium spp.* gives negative results in catalase test. (UK Standards for Microbiology Investigations, October 10, 2011).

According to Cappuccino and Sherman (2004), a *Bacillus sp* will show starch hydrolysis, gelatine liquefaction and lipid hydrolysis. *Bacillus* culture streaked on Simmon Citrate Agar showed growth on the slants as well as changing the media from blue to green (Beishir 1991).

Most of the *Bacillus spp.* ferment carbohydrates particularly glucose, sucrose and mannitol, but not all *Bacillus spp.* ferment all the sugars, for example glucose is usually fermented by *Bacillus subtilis* and mannitol by *Bacillus megaterium*, thus this helps in identifying the particular species of *Bacillus*. Lactose is usually not fermented by *Bacillus spp.* (Aneja, 2014).

Tests like Methyl red test and Indole production tests are usually done to differentiate between the members of family *Enterobacteriaceae* (Aneja 2014), and since all the colonies showed negative results, and are gram-positive, they do not belong to family *Enterobacteriaceae.*

According to MCB 301 Lab Manual Supplemental Spring 2008, VP test is positive for *Bacillus subtilis*.

Liu and Jurtshuk (1986) studied that out of 293 *Bacillus* strains analyzed 23% were oxidase positive, 47% were oxidase indeterminate and 30% were oxidase negative so different species in Genus *Bacillus* may give different results, for example *Bacillus subtilis* gives oxidase positive results, while others like *Bacillus cereus* may give oxidase negative results.

Thus, the biochemical analysis indicates that our test organisms labelled 1, 2, 3 and 4 belong to genus *Bacillus*, and test organism 5 belonged to *Brevobacillus* sp while test organism 6 belonged to *Lysinobacillus* sp. and the species can be identified using ABIS online tool. Finally, molecular analysis using 16s rRNA sequencing identifies Strain 1 as *Bacillus subtilis*, Strain 2 as *Bacillus cereus*, Strain 3 as *Bacillus mycoides*, Strain 4 as *Bacillus thuringenesis*, Strain 5 as *Brevobacillus parabrevis* and Strain 6 as *Lysinibacillus fusiformis*. Thus, 4 isolates were identified as *Bacillus sp.* and shows the dominance of *Bacillus sp* in the sample.

5. Conclusion

The present study showed the presence of Bacillus sp. in the wastes obtained from Nagaon Paper Mill. These wastes may turn useful as they may become the raw materials for the pharmaceuticals and other industries which require lots of bacteria for the production of various antibiotics, enzymes, pesticides and genetically modified organisms. Thus, the wastes would be recycled and the manufacturing costs of industries would decrease. However, proper isolation and identification should be done to get the right organisms.

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