The legacy of bio-molecules as a bio-fertilizer: Context of single cell fertilizer Isolation and partial characterization of an amylolytic bacterium

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Repeated cultivation of crop plants is the reason of depletion of nutrient in an agricultural land. Therefore, modern procedures of agriculture cascaded with addition of organic and inorganic fertilizers, use of insecticides and pesticides, addition of proper water etc. Various inorganic molecules are used as fertilizers. However, use or organic manures are also in practice. They have many roles such as they improve soil porosity, air holding capacity, water holding capacity, structure, texture etc. Agricultural scientists suggest using much amount of organic molecules because of many reasons. Bio-fertilizers of many kinds are used by farmers of all nations. However, these fertilizers are unable to show tremendous effects on growth and development of crop plants. Even though, these fertilizers have cumulative effects. The present work focuses on use of bio-molecules as bio-fertilizer. To make these molecules an amylolytic bacterium was isolated and partially identified based on microscopic observations and biochemical tests. The optimum pH, temperature, substrate concentration etc were studied. The optimum pH and temperature for the growth of the isolate were pH 7.0 and 37.0°C respectively. However, the organism grows even in 60.0°C. The organism uses four commonly available natural substrates as carbon source. Among them potato starch is most conveniently utilized by the organism. The amy gene of the strain was cloned using a vector. It expressed high amount of amylase (data is not shown). The recombinant organism was used to make bio-molecules. It was grown in presence of various natural substrates and enzymatic activities and other associated studies were also carried out. The experimental results obtained in this study showed that the recombinant organism can be utilized to make huge amount of bio-molecules. It will be a unique fertilizer for the future generation.

Keywords: Single cell fertilizer (SCF), Bio-molecules, Bio-fertilizer, Agricultural land, Amylase, Bacterial strain

Introduction:

Use of fertilizer is pivotal to obtain bumper crop (Cooke, 1969, Banerjee et al. 2010). Fertilizers that are used in agriculture can be grouped into two categories. In the first category various inorganic molecules that are produced in industries such as sodium nitrate, ammonium sulphate, urea and others (Khandalwal, Singh and Kapoor 1977). The second group is made of various organic molecules such as decomposed organic materials, compost and bio-fertilizers of many kinds (Vacchani and Murty, 1964, EI-Hawary et al 2002). Agricultural scientists suggest use of various kinds of organic molecules instead of inorganic molecule because organic molecules have various effects on agricultural land. They ensure good health of an agricultural land because they increase soil structure, texture, air as well as water holding capacity (Jeyaraman and Purushothaman, 1988; Kannaiyan, 1990a). The major disadvantage of use of inorganic molecules is that they contaminate underground water and deposit in food materials (Gasser, 1964; Hutchinson and Viets, 1969). The term “Green revolution” is used for bumper and sustainable production of plant based food material. Many agricultural scientists suggest use of bio-fertilizer (Subba Rao, 1993) such as *Azotobacter* (Mishustin and Shilnikova, 1969; Shende and Apte, 1982), *Rhizobium* (Burton, 1979; Keyser, Somasegaran and Bohlool, 1992), Blue green algae (De, 1993; Boussiba, 1991), *Azolla* (Lumpkin and Plucknett, 1980; Gopalaswamy et al., 1994), VAM fungi (Abbot and Robson, 1982; Bolan, 1991) etc. These organisms are able to fix nitrogen however they intake carbon, phosphorus and minerals from soil. Blue green algae and ferns (Ashton and Walmsley, 1976) are able to fix carbon dioxide and nitrogen. However they intake minerals from soil (Sen et al., 2009). Therefore, they are in competition for food and nutrition in an agricultural land (Sen, 2007). In this communication, an amylolytic bacterium is isolated and partially characterized by screening of huge number of soil samples. The organism is grown in the presence of various natural substrates. Degraded product of bacterial cell can be utilized as potent organic fertilizer. The effectiveness of these organic molecules as an organic fertilizer is reported (Sen et al. 2009) however field experiments are yet to be carried out. Moreover it is the first effort to produce huge amount of biomass using an amylolytic bacterium. .

Materials and Method:

Soil samples were collected from various places of the district Burdwan, West Bengal, India. These soil samples were used to isolate amylolytic bacterial organisms. Following chemicals were purchased from the supplier indicated. Glucose, soluble starch, beef extract, KH_2PO_4, K_2HPO_4, NaCl, KI, Agar-agar were from Merck, Germany, India. Dinitro salicylic acid (DNS) was purchased from Loba Chemicals. Other materials which were used in this study were from various chemical suppliers. *Escherichia coli* strains of many kinds were the gifts of Dr. T. K. Dutta, Dr. Sujoy Dasgupta of Bose Institute, Kolkata and Dr. H. K. Majumder of Indian Institute of Chemical Biology, CSIR, Jadavpur, Kolkata. These bacterial strains were used in this study.

Screening of amylase producing bacteria: 1.0 g of soluble starch was mixed with 0.1g of beef extract, 0.5g of K_2HPO_4, 0.5g of KH_2PO_4 and 0.5g of NaCl in100 ml distilled water. The pH was adjusted to 7.0 with the addition of requisite amount of 0.5N NaOH or 0.5N HCl. After the adjustment of pH of the medium 1.5% agar-agar was added. The sterilized medium was used to make Petri plate. Dilute soil samples were spreaded onto the surface of a Petri plate containing the medium and incubated at 37^0C. The bacterial colonies which were appeared on the Petri plate were transferred. Colonies were purified either by dilution or by streaking procedures. The purified bacterial colonies were preserved separately in slants containing the same media.

Assay of amylase activity: The amylase activity of 100 bacterial strains was done by growing the organisms in starch agar plate and staining with iodine and KI solution. The strains those produced broad starch hydrolysing zone were selected for further studies. Amylase activity was assayed following the procedure described by Bernfeld (1955) with a little modification. 1.0 ml of 1.0 % starch solution was used as substrate , 100mM Sodium phosphate buffer pH 7.0 was added in the reaction mixture. 0.2 ml of culture filtrate was used as the source of the enzyme. The final volume of the assay was adjusted to 2.0 ml using requisite amount of distilled water. The reaction mixture was incubated at 37^0C for 15 min. After the incubation period, 3.0 ml of DNS was added. The sample was heated at 100^0C for 10 min. The amount of reducing sugar produced from the substrate was calculated from the standard curve which was made using glucose. 1 unit of enzyme activity was µ mole of glucose produced by the enzyme/ml of culture filtrate in the standard assay conditions.

Growth of the organisms using various natural substrates: Five bacterial isolates which showed highest amylase activity were used for further studies. Various natural substrates such as potato, tomato, pumpkin, cucumber and bottle-gourd tissues were used in the medium. 5.0 %.of tissue extract was used in the medium and other ingredients were remained same as described. The medium was inoculated with respective isolate and incubated in shake condition at 37^0C for 20 hrs. The growth of each isolate was measured at 540 nm using a colorimeter. The organism which showed maximum growth in the medium containing natural substrate was selected for the production of bio-molecules.

Partial identification of the organism: The organism was grown on starch medium. The morphological characterization was carried out by Gram staining. Various biochemical tests were carried out following Bergey's Manual of Determinative Bacteriology such as IMViC test, nitrate reduction test, triple sugar iron test, catalase test, mannitol motility test, Phenylalanine degradation test, Oxidation fermentation test, Gelatin hydrolyzation and utilization of different carbon sources.

Determination of optimum pH and temperature of the growth of the strain, AB -56: 1.0 % soluble starch was used in the medium. Other ingredients were remained same as described before. 50.0 ml of this medium was taken in 250 ml conical flask and the pH was adjusted separately to 1.0, 3.0, 5.0, 7.0, 9.0 and 11.0 respectively. The medium was sterilized, inoculated and incubated at 37 ^{0}C in shake condition for 18 hrs. The strain was also grown in various temperatures using the same medium, pH 7.0. The temperatures were 17.0 ^{0}C, 27.0 ^{0}C, 37.0 ^{0}C, 47.0 ^{0}C, 57.0 ^{0}C and 67.0 ^{0}C respectively. The bacterial growth was measured at 540 nm using a colorimeter.

Results:

Isolation of amylolytic bacteria: Amylolytic bacterial strains were isolated from soil by spread plate method using serial dilution of soil samples collected from the agricultural lands of the district Burdwan, West Bengal, India. The isolated bacterial strains were purified and grown separately in starch plates. The plates were then stained with I_2 and KI solution. The bacterial colonies that shown high hydrolyzing zone were selected. The extra-cellular amylase activity of these isolates was also determined following standard procedure. The

isolates that showed higher amylase activity were grown in presence of various natural substrates.

Utilization of various natural substrates: 5 isolates were used in this study that showed higher amylase activity. These isolates were grown in presence of 5.0 % potato, tomato, pumpkin, cucumber and bottle-gourd in shake condition at 37.0 ^{0}C. The growth of the organisms was measured at 540 nm. (Table.1). The bacterial strain AB-56 was used for further work as it utilized maximum amount of natural substrate and produced highest amount of extra-cellular enzyme.

Table.1. Growth of selected bacterial isolates in presence of natural substrates *

Bacterial strain	Natural substrate				
Strain no.	**Potato**	**Pumpkin**	**Tomato**	**Cucumber**	**Bottle-gourd**
AB-13	0.27	0.3	0.07	0.19	0.16
AB-27	0.27	0.26	0.25	0.19	0.09
AB-55	0.28	0.25	0.17	0.2	0.04
AB-56	0.56	0.52	0.41	0.29	0.08
AB-63	0.35	0.51	0.36	0.28	0.08

* the optical density was taken at 540 nm , incubation period 20 hrs, temperature of incubation 37.0 ºC , grown in shake condition, other conditions were same as described in materials and methods.

Characterization of isolated bacterial strain AB -56: The bacterial strain AB-56 (Fig.1) was selected as it showed maximum capability of utilization of natural starch. The organism was stained using crystal violet and safranin. The Gram stain showed that the organism took crystal violet color. Therefore the organism is Gram positive. The microscopic observation showed the morphology of the bacteria. The organism is a rod shaped, Gram positive (Fig.2). Approximate size of the organism was 2.1µm X 3.5µm. Fig. 1 showed the growth of the organism in starch agar medium.

Fig.1. Bacterial strain AB-56 was grown on agar plate containing starch.

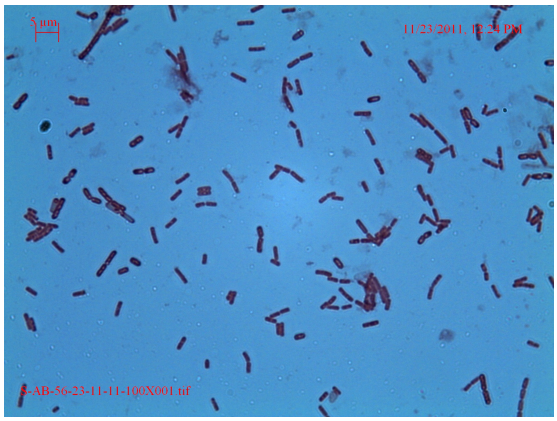

Fig.2. Gram staining of AB-56

Some biochemical tests were also performed according to Bergey's manual of determinative bacteriology. These were IMViC test, Nitrate reduction test, Triple sugar iron test, catalase test, Mannitol motility test, Phenylalanine degradation test, Oxidation fermentation test, Gelatin hydrolyzation (Table.2). The organism is IMViC test negative that's mean it cannot produce indole or organic acid or neutral products and it cannot utilize citrate. The organism cannot reduce nitrate or it cannot produce H_2S in Triple sugar iron test. The organism also shows negative result in Mannitol motility test. The organism is catalase test positive that's mean it can degrade H_2O_2 into water and oxygen. The organism can degrade phenylalanine and produce Phenyl pyruvic acid which can be detected by addition of ferric chloride. The organism also shows gelatin hydrolysis and it shows fermentative reaction in Oxidation-Fermentation test.

Table.2. Result of biochemical tests performed using bacterial strain AB56

Biochemical test	**Result**
Indole test	Negative
Methylene test	Negative
Voges-Proskaur Test	Negative
Citrate Utilization Test	Negative
Nitrate Reduction Test	Negative
Triple Sugar Iron Test	Negative
Mannitol motility Test	Negative
Catalase Test	Positive

Phenylalanine Degradation Test	Positive
Gelatin Hydrolysis	Positive
Oxidation-Fermentation Test	Positive

The carbon utilization showed that the organism utilizes glucose, ribose, sucrose,lactose, maltose, mannitol ,CMC and starch. However, the growth of the organism was variable in presence of these carbon sources. The growth was moderate in presence of sucrose, lactose and ribose. The growth was inhibited in presence of cellulose (Table.3). The optical density was measured at 540 nm.

Table.3. Different carbon source utilized by the bacterial strain AB56

Carbon source	O.D at 540 nm.
Glucose	1.33
Ribose	0.67
Sucrose	0.83
Lactose	0.71
Maltose	1.08
Mannitol	1.52
Strach	1.66
Carboxy methyl Cellulose	0.28

Growth of the organism in various pH and temperature: The isolated strain was grown in presence of variable pH. The pH used in this study were pH 1.0, 3.0, 5.0, 7.0, 9.0 and 11.0 respectively. It showed that the bacteria grown maximally at pH 7.0 (Fig.3). Below and above this pH it showed inhibitory effects.

The organism was grown in presence of various temperatures. These were 17.0 ^{0}C, 27.0 ^{0}C, 37.0 ^{0}C, 47.0 ^{0}C, 57.0 ^{0}C and 67.0 ^{0}C respectively. The maximum growth was obtained at 37.0 ^{0}C. However the organism also showed growth at high temperature. It showed growth even at 60.0 ^{0}C. It was not less bacterial cell compare to that of 37.0^{0}C which was the optimum temperature for growth of the organism (Fig.4).

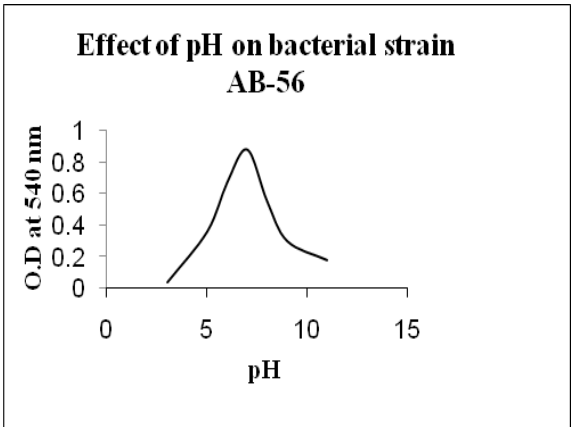

Fig.3. Effect of pH on bacterial strainAB56. It shows optimum pH is 7.0.

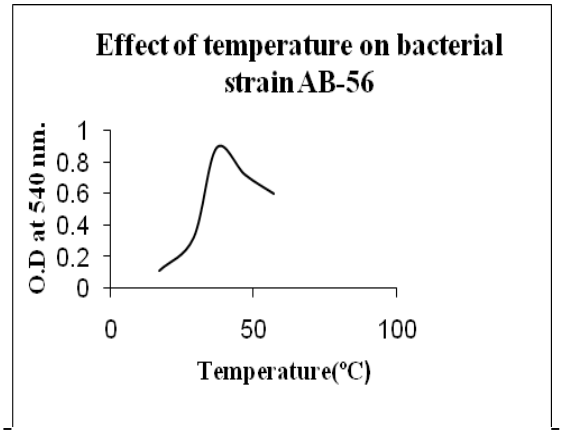

Fig.4. Effect of Temperature on bacterial strain AB56. It shows optimum temp. is 37.0^0C.

Extracellular amylase activity of the bacterial strain AB56: The organism was grown in presence of 1% soluble starch at 37^0C in shaking condition and at pH 7.0. The culture filtrate was used as the source of enzyme. The reducing sugar which was produced in the assay was calculated using a standard curve made using glucose. The extracellular enzyme activity of the organism was 14.82 units in standard assay condition. The optimum conditions for the enzyme assay were also determined. The optimum pH was at 5.0 and optimum temperature for the enzyme activity was at 37.0 ^{0}C , using the standard assay procedure the Vmax and Km value were determined , effect of various metal ions on enzyme activity were also carried out (data is not shown).

Discussion:

Nitrogen fertilizers are used abundantly in agriculture because of tremendous effect of these fertilizers on growth and development of crop plants (EI-Zeiny et al., 2001; Gorttappeh et al., 2000). It is also reported that these fertilizers are able to increase the yield of various crop (Aly, Soliman, Etakel and Alinit, 1999). The present study wishes to use bio-molecules as organic fertilizer. These bio-molecules are produce in a bacterial system. Instead of intact bacterial cell the released molecules from an amylolytic bacterium is utilized as organic fertilizer (Sen, 2007). In this study a bacterium is isolated from soil sample collected from Burdwan district, West Bengal, India. It is able to produce extracellular amylase. The organism is a Gram positive bacillus. Various biochemical tests were also carried out to characterize the organism. However proper characterization of the organism is yet to be carried out. The strain is an amylolytic organism. Various amylolytic bacteria are reported by

various investigators. Their enzymes are characterized, some of them are also crystallized. Molecular cloning and expression the gene are also been reported by several investigators (Sen and Oriel, 1989). The organism is a thermotollerant organism because it grows at high temperature. However the enzyme showed maximum activity at 37.0 ^{0}C. The pH optimum was also determined. It was pH 5.0. Other investigators are also reported similar kind of result. The organism was grown in presence of various natural substrates. In presence of potato starch the bacteria showed highest growth. However the organism is able to utilize other organic materials used in this study. The present work studied the optimum pH and temperature of the extracellular enzyme. The work is also extended to clone the gene and its expression in a bacterial host (*E.coli* DH5α). The work was to make bacterial cellular molecules using potato starch as carbon source. The bacterial cellular molecules will be isolated from the intact bacteria by treating with virulent bacteriophage. The work desire to use these molecules as organic fertilizer because whenever various intact bacteria are used as bio-fertilizer the molecules remain inside the cell. These molecules will not be available to the plant until degradation and death of the bacterial cell. However the presence study used these molecules as organic fertilizer. Various investigators use intact bacterial cell like *Azotobacter*, *Rhizobium*, blue green algae, *Azolla* as bio-fertilizer (Tesar, 1984; Sinha, 2003). These fertilizers are unable to show desirable growth and development of crop plants because these molecules remain inside the intact bacterial cell. Therefore farmers do not like to use bio-fertilizer even though they do have cumulative effect.

Acknowledgements:

We are thankful to Mr. R.N. Majumdar, Chairman of Rahul foundation for encouragement and providing infrastructure to do the work at the laboratory of DCCS, Rajbandh, Durgapur. AB is the JRF of DST project, Govt. of West Bengal. We are thankful to DST-West Bengal, India for financial support. We are also thankful to all teachers and non-teaching staffs of DCCS.

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