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Cloning and expression of an amylase gene from *Bacillus* sp. isolated from an agricultural field in West Bengal, India

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The use of fertilizer is pivotal to ensure soil fertility. Green fertilizers have many advantages and bio-molecules generated in a bacterial cell that can be used as green fertilizer. To produce bio-molecules from a bacterial cell, a bacterium was isolated that uses natural starch as sole carbon source. The organism was grown in presence of various natural starchy materials. The organism was partially identified as *Bacillus* sp. The optimum conditions for growth of the strain were: temperature of 37.0°C and pH 7.0. However, it grows well even above 55.0°C. The optimum conditions for degradation of various starchy substrates using extra-cellular amylase of the strain were studied. The optimum pH was 4.0 for soluble starch whereas it was pH 5.0 for other substrates and temperature was 17.0°C for soluble starch and 37.0°C for others. Prolongation of time of incubation, velocity of enzyme reaction, and optimum concentration of various metal ions for amylase activity was also studied using optimum assay conditions. Molecular cloning and expression of amy gene of the strain was done in *Escherichia coli* DH5α. The host cell harboring the recombinant plasmid was grown in the presence of potato starch and incubated for many days to obtained cellular lysate and the liquid biomass might be used as bio-fertilizer.

Key words: Amylolytic bacteria, amylase, bio-molecules, bio-fertilizer, single cell fertilizer.

INTRODUCTION

Enormous use of inorganic fertilizers (Prasad et al., 2004; Tiwari, 2007; Tandon, 2010), insecticides, and pesticides (Pimentel, 1995) is the modern technique of agriculture. Inorganic fertilizers, used in agriculture, deposit in food materials and can also cause underground water pollution (Nolan et al., 1997; McIsaac, 2003). Bio-

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fertilizers have multiple advantages over the inorganic fertilizers (Prasanna et al., 2008; Sheoran et al., 2010).They improve soil structure, texture, porosity, air holding and water holding capacities (Prasad, 1998). Bio-fertilizers are nitrogen fixing bacteria, blue green algae, ferns etc (Kennedy et al., 2004; Mishra and Pabbi, 2004; Baset Mia and Shamsuddin, 2010). As they are living cells they compete for food and nutrition with the crop plants, even though, the competition is incipient (Sen, 2007), and therefore *in vitro* production of various bio-molecules from a bacterium and using them as bio-fertilizer is the theme of the present study. Bacteria have less generation time; therefore huge amount of bio-mass can be generated using fermentation technology. Single cell fertilizer (SCF) is made of cellular molecules such as proteins, free amino acids, carbohydrates, free sugars, lipids, free fatty acids, nucleotides, macro and micro elements, and others (Sen et al., 2009). Various molecules that are present in SCF will be absorbed by crop plants as readymade food for their growth. The main object of this study is the production of SCF from a bacterium that can utilize low cost carbon source for its growth. The present study includes isolation of an amylolytic bacterium, partial characterization of the organism, partial characterization of its extra-cellular enzymes, molecular cloning and expression of the amy in *Escherichia coli* host and use of the recombinant strain as the source of SCF. Furthermore, it is the first report on production of SCF using a recombinant bacterium.

MATERIALS AND METHODS

Soil samples were collected from various agricultural fields of the district Burdwan, and other districts of West Bengal, India. These soil samples were collected from 5 to 10 cm depth during the month of March 2011. Temperature was around 30.0 ± 2.0°C. The following chemicals were purchased from the suppliers indicated: soluble starch, glucose, DNS, NaOH, KOH, KH_2PO_4, K_2HPO_4, NaCl etc. were from Merck, India; other chemicals such as phenol, chloroform, ethyl alcohol etc. were from Glaxo Smith co., Mumbai, India; restriction endonuclease, T-4 DNA ligase, and alkaline phosphatase were purchased from GeNei, Bangalore, India.

Isolation of an amylolytic bacterium

Starch medium was prepared by using 1.0 g of starch, 0.5 g NaCl, 0.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.01 g beef extract in 100 ml distilled water, at pH 7.0. Using dilution plate technique various mesophilic amylolytic bacteria were isolated. Among them, those that showed higher extra-cellular amylase activity were grown in the presence of different natural starchy materials. The organism which showed highest growth in presence of natural starch was selected for further studies.

Partial identification of the organism

The organism was grown on potato starch medium. The morphological characterization was carried out by Gram staining and electron microscopic observation. Electron microscopy of potato starch granules that was used in the medium was also studied. Endospore staining was performed following standard method (Schaeffer and Fulton, 1933). Various biochemical tests were carried out following Bergey's Manual of Systematic Bacteriology (2009). Extracellular and intracellular amylase activities in presence of different carbon source were also determined.

Optimization of pH, temperature and potato starch concentration for growth of the bacterium

Standardization of pH, temperature and substrate concentration for optimum growth of the bacterium were carried out. 1.0% potato starch was used in the medium with other ingredients as described before. 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 20 h. The bacterial growth was measured at λ=540 nm using a colorimeter (Klett colorimeter, Clinical model: 800-3, 115 VAC). Determination of optimum temperature was also carried out using same medium. The incubation temperatures were 17.0, 27.0, 37.0, 47.0 and 57.0°C, respectively. The conditions of growth were determined. Different concentrations of potato starch (0.5, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0%) were used in the medium to determine the optimum substrate concentration. After incubation at 37.0°C for 20 h, O.D was taken.

Assay of amylase activity of the isolated strain

Isolation of extracellular enzyme

The organism was grown using optimum condition for 20 h in a shaker. The culture was then centrifuged at 5,000 rpm at 4.0°C for 10 min. The clear supernatant was used as the source of extra-cellular enzyme.

Assay of amylase

The assay followed a standard protocol for sugar estimation (Bernfeld, 1955). The assay was carried out using 1.0 ml of 4 different substrates (1% soluble starch, potato, rice, and wheat starch), 0.5 ml of 100 mM phosphate buffer and 0.2 ml of extra-cellular enzyme. The final volume of the reaction mixture was adjusted to 2.0 ml using requisite amount of distilled water (Sen and Oriel, 1989a) for 15 min at optimum temperature. The reducing sugar which was produced in the assay condition was estimated using a colorimeter at λ=580 nm. One unit of enzyme activity was described as µmole of reducing sugar (as glucose) produced per ml of the extra-cellular enzyme per min. Effects of pH, temperature, prolongation of time of incubation, substrate concentration and effect of different metal ions on extracellular amylase activity against four substrates were also determined. PH optimum was determined using buffer with variable pH like 1.0, 3.0, 4.0, 5.0, 7.0, 9.0 and 11.0, respectively. The buffers were prepared according to Gomori (1955).

Different temperatures like 10.0, 17.0, 27.0, 37.0, 47.0, 57.0, 67.0 and 77.0°C were used to determine temperature optimum. Effect of prolongation of time of incubation was carried out by incubating the assay mixture at various time periods. The enzyme activity was also recorded using various substrate concentrations. The concentrations were 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0 and 50.0 mg, respectively. K_m and V_{max} value against four substrates were also determined. Different monovalent and divalent

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

Strain number	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
AB-65	0.37	0.44	0.37	0.21	0.10
AB-66	0.23	0.34	0.26	0.14	0.06
AB-67	0.28	0.32	0.26	0.23	0.07
AB-88	0.41	0.21	0.22	0.21	0.08
AB-98	0.37	0.38	0.26	0.29	0.19

cations like Na^+, K^+, Mn^{2+}, Ca^{2+}, Mg^{2+} and Zn^{2+} were used to determine the effect of concentration of metal ions on enzyme activity.

Molecular cloning and expression of the 'amy' of the bacterium in an *E. coli* host

Molecular cloning and expression of amy gene of the organism in *E. coli* host were carried out following standard procedures with a little modification (Sen, 2006). The genomic DNA of the strain was isolated and purified using standard procedure (Green and Sambrook, 2012). Approximately, 50.0 µg of genomic DNA was completely digested by using 10 units of Hind III restriction endonuclease for 8 h at 37.0°C. Then pBR322 plasmid DNA from *E. coli* HB101 strain was isolated and purified. About 20.0 µg of purified plasmid DNA was digested with 5.0 units of Hind III restriction endonuclease for 8 h at 37.0°C. It was extracted with TE saturated phenol, chloroform and precipitated with ethyl alcohol. The plasmid was then treated with 5.0 units of alkaline phosphatase for 8 h at 37.0°C. The ligation of Hind III digested genomic DNAs and alkaline phosphatase treated plasmid DNA was carried out using equal molar concentrations of plasmid and genomic DNAs in 50.0 µl of ligation mixture. The ligation was carried out by using 4.0 units of T4 DNA ligase at 21.0°C for 48 h. The transformation was carried out using 100.0 µl of competent cells (*E. coli* DH5α) and 10 µl of ligated mixture. After the transformation, the cells were incubated using 1.0 ml of Luria broth for 24 h. Then cells were plated using starch agar plate containing 35.0 µg/ml of ampicillin.

Data analysis

Scientific data analysis and graphing was done following Sigma plot 12 scientific graph system. Microsoft office excel 2007 was also used to make various graphs. Other results were expressed as mean value.

RESULT

Isolation of an amylolytic bacterium from soil sample

Starch degrading, extracellular amylase producing bacteria were isolated by spread plate method. To detect starch degradation, plates were flooded with I_2 and KI solution. The colonies that showed higher zone of starch hydrolysis were selected. These were then grown in 1% starch containing broth and growth was measured at 540 nm. The isolates which showed higher growth rate were grown in presence of different natural starchy materials like potato, pumpkin, tomato, cucumber and bottle-gourd (5%) and incubated at 37.0°C in shake condition for 20 h. It showed that the ability of using natural starches was highest for the strain AB-56 (Table 1).

Partial characterization of the strain AB-56

The strain AB-56 showed highest growth in presence of natural carbon sources and it utilized potato starch more conveniently. Morphological characters were observed by Gram staining (Figure 1c) and also by scanning electron microscope (Figure 1a). Endospore staining (Figure 1d) was carried out for conformation of spore formation. Electron microscopic study of starch granules was also carried out (Figure 1b). Many biochemical tests were performed to identify the organism (Table 2). From the above tests and other associated experiments, the organism was partially characterized as *Bacillus* sp., utilization of different carbon sources, extracellular and intracellular amylase activity in presence of those carbon sources is presented in Table 3.

Determination of optimum growth conditions of the strain AB-56

Standardizations of pH, temperature and substrate concentration for optimum growth of the organism were carried out. It showed that the maximum growth of the organism was at pH 7.0 (Figure 2a) above or below this pH it showed inhibition of the growth of the organism. Determination of optimum temperature was carried out by incubating the culture medium at various temperatures

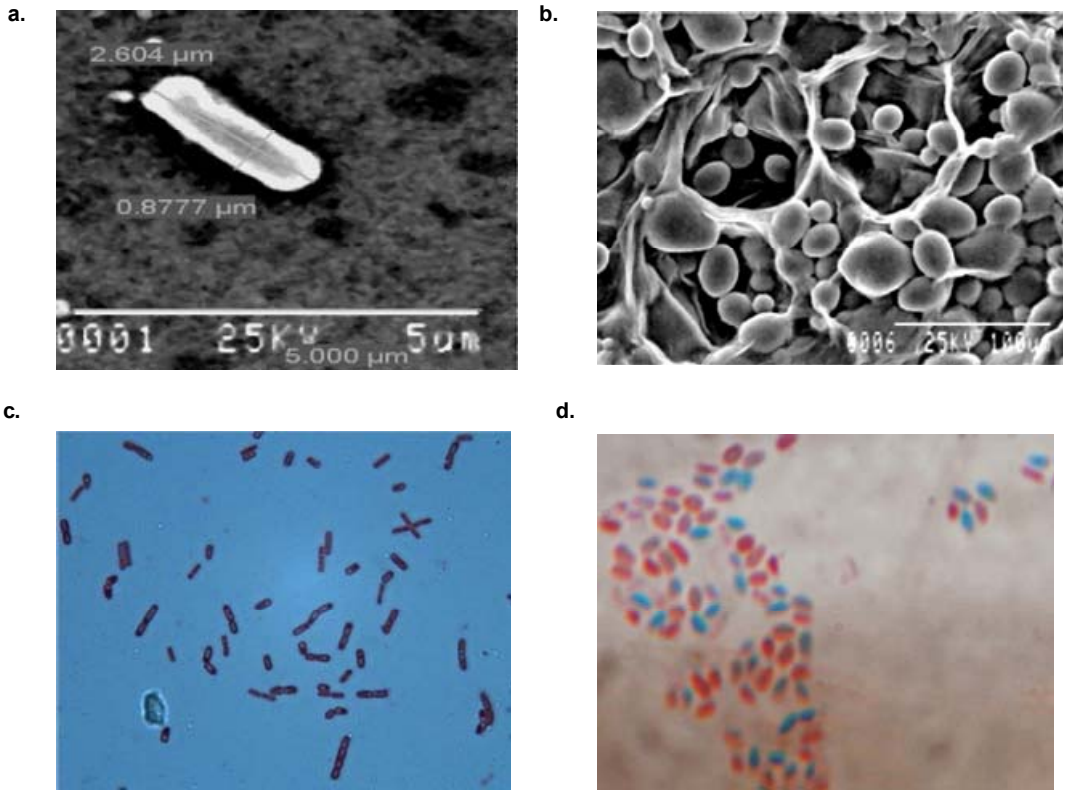

Figure 1. Morphological study of the strain AB-56 and potato starch granules**. a)** Scanning electron microscopic view of the organism. **b)** Scanning electron microscopic view of potato starch granules used to grow the bacterial strain. **c)** Light microscopic view of the organism after gram staining. **d)** Green colours spores after endospore staining.

Table 2. Characteristics of the strain AB-56.

Test	Result
Strain characteristics	
Shape of the colony	Rod shape with irregular edge
Colour	Slightly yellowish orange
Gram character	+
Spore	+
Size	2.6 µm × 0.9 µm
Biochemical characteristics	
IMViC test	–
Nitrate reduction test	–
Triple sugar iron test	–
H_2S production test	–
Catalase test	+
Urease test	–
Mannitol motility test	–
Phenylalanine degradation test	+
Tolerance of NaCl	4%
Oxidation fermentation test	+
Gelatin hydrolization	++

Table 3. Effect of different carbon sources on growth of AB-56 and induction of extracellular or intracellular amylase by the same.

Carbon source	Growth	Units of extracellular amylase activity	Units of intracellular amylase activity
Glucose	1.33	Nil	Nil
Ribose	0.67	Nil	3.7
Sucrose	0.83	Nil	4.65
Lactose	0.71	Nil	3.7
Maltose	1.08	Nil	752.
Mannitol	1.52	15.7	3.7
Starch	1.66	17.6	6.48
CMC	0.28	13.88	Nil

1% carbon source was used in the medium. 100 µl of cell lysate was used for intra-cellular amylase assay.

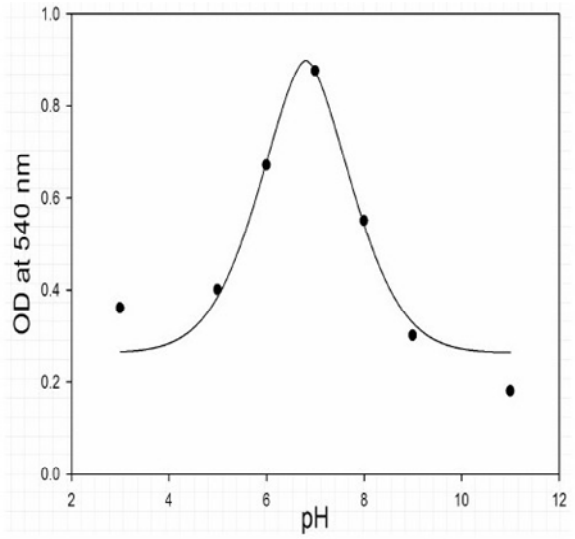

Figure 2. Determination of optimum pH and temperature for the growth of the strain. **a)** Effect of pH on the growth of AB-56. **b)** Effect of temperature on the growth of AB-56.

Table 4. Effect of potato starch concentration on the growth of AB-56.

Potato starch (%)	Growth at 540 nm
0.5	0.24
2.0	0.30
4.0	0.48
6.0	0.69
8.0	0.88
10.0	0.40
12.0	0.34

with pH 7.0. It showed the maximum growth of the organism at 37.0°C (Figure 2b). Growth of the organism was also recorded at 57.0°C where the O.D was 0.60 which represents its capability to grow at high temperature. The strain AB-56 was grown in presence of variable starch concentrations where potato starch was used as sole carbon source (Table 4). It showed that the optimum growth occurred when potato starch concentration was 8.0% and above or below this concentration there were reduction of growth.

Extra-cellular amylase activity of the strain AB-56

Effect of pH on amylase activity

The optimum pH for amylase activity was carried out using four different substrates. The result showed that when soluble starch was as substrate, the optimum pH was 4.0 while for other substrates it was 5.0 (Figure 3a).

Effect of temperature on amylase activity

The optimum temperature for amylase activity was also determined using those substrates. When soluble starch

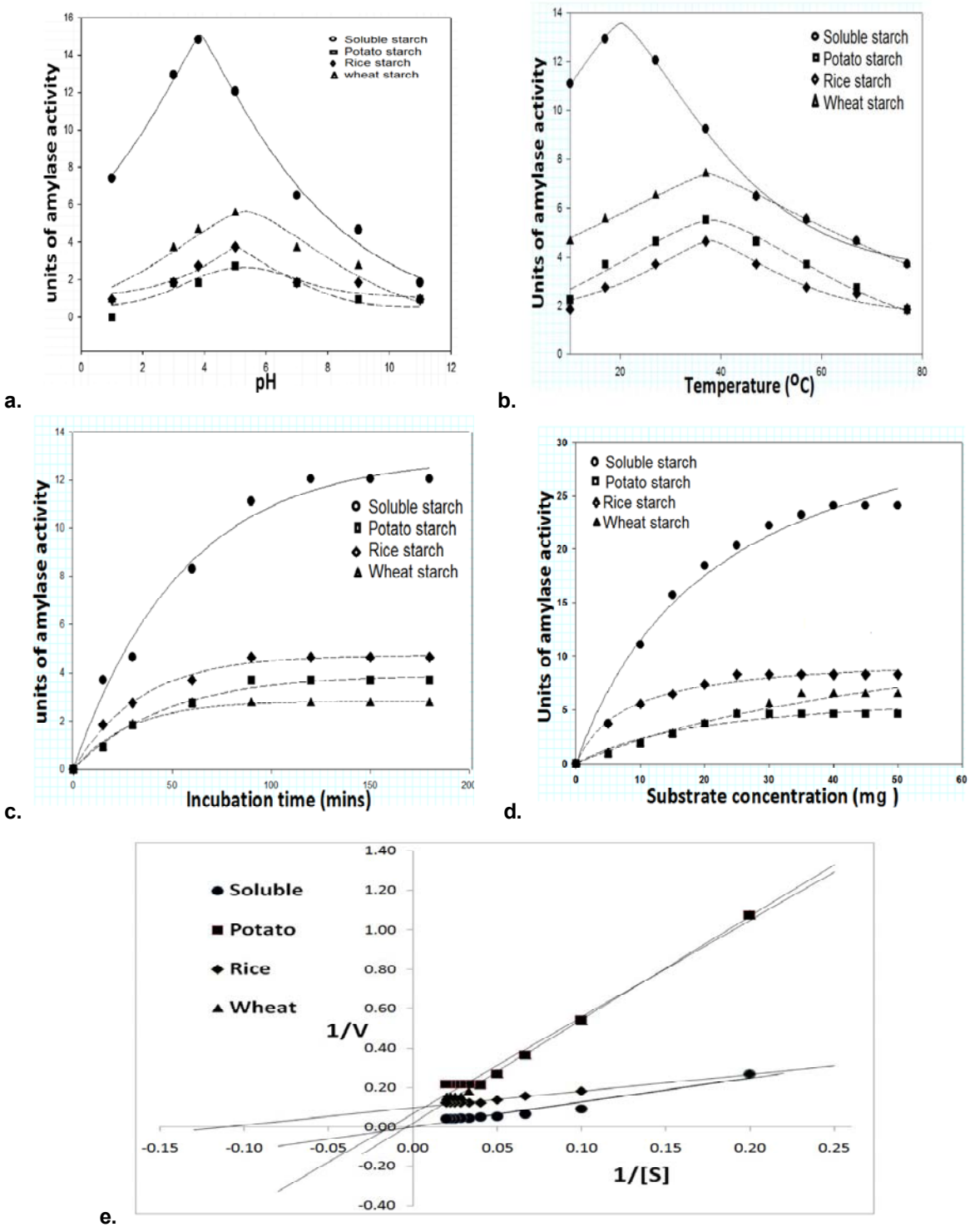

Figure 3. Effect of different parameters on extracellular amylase of the strain AB-56. **a)** Effect of pH on amylase activity. Four different substrates were used. 100 mM buffer of different pH were used in these assays. **b)** Effect of temperature on amylase. Different temperatures were used and other parameters were remained same as described before. **c)** Effect of prolongation of time of incubation period on amylase activity. The reaction mixture was incubated in various time periods using standard assay conditions. **d)** Effect of substrate conc. on amylase activity. Various concentrations of substrate and standard assay conditions were used. **e)** Double reciprocal plot of substrate concentration. [S] v/s (V) plot of amylase showing the effect of substrate concentration on the velocity of amylase activity.

was used as substrate it showed optimum temperature at 17.0°C while for other substrates it was at 37.0°C (Figure 3b).

Effect of prolongation of time of incubation

Amylase activity was measured against prolongation of time of incubation. These assays were carried out using optimum pH and temperature. When soluble starch was used as substrate, the amylase activity was increased upto 150 min of incubation but for other substrates, it was 90 min. Further prolongation of incubation time showed no change in enzyme activity (Figure 3c).

Effect of substrate concentration on amylase activity

Effect of substrate concentration on amylase activity was measured using various substrate concentrations. Hydrolysis of starch was increased linearly with increasing substrate concentrations and reached a plateau at 45.0 mg concentration for soluble starch. Similarly for potato, rice, wheat starches it was 35.0, 25.0 and 40.0 mg, respectively. It produced an apparent Km value of 110.6, 110.6, 38.8 and 313.65 mg/ml, and Vmax of the enzyme were 12.32, 2.45, 3.35, 5.33 µmoles/min/ml for soluble, potato, rice and wheat starches, respectively (Figure 3d). Lineweaver burk plot also produced similar results (Figure 3e).

Effect of various metal ion concentrations on amylase activity

Various concentrations of monovalent, divalent cations like Na^+, K^+, Ca^{2+}, Mn^{2+}, Mg^{2+} and Zn^{2+} were used in these assays. These ions were added in the form of salts of chloride or sulphate. The concentrations of cations used in the reaction mixture were 1.0, 5.0, 10.0, 20.0, 40.0 and 60.0 mM, respectively. But in case of Zn^{2+} the concentrations were 1.0, 2.0, 3.0, 4.0, 5.0 mM, because higher concentrations produced precipitation. In the presence of Na^+ ion maximum amylase activity was at 1.0 mM concentration for all substrates. Above this concentration, enzyme activity was decreased (Figure 4a). 5.0 mM K^+ was the optimum concentration for soluble starch and for other substrates it was 1.0 mM (Figure 4b). In presence of Ca^{2+} ion the optimum enzyme activity was at 1.0 mM concentration for soluble starch, but for others the maximum enzyme activity was obtained at 10.0 mM concentration (Figure 4c). When Mn^{2+} was used in the reaction mixture, for soluble starch enzyme activity gradually increased but the optimum concentration for potato starch was 40.0 and 30.0 mM, respectively (Figure 4d). The optimum concentration of Mg^{2+} was 1.0 mM for all substrates however in presence of potato starch it showed no effect (Figure 4e). The maximum enzyme activity was obtained at 1.0 mM concentration in presence of all four substrates for Zn^{2+} ion. Above this concentration it showed inhibitory effects (Figure 4f).

Molecular cloning and expression of the 'amy' of AB-56 in an *E. coli* host

The genomic DNA was isolated, purified and cleaved with Hind III restriction endonuclease. The Hind III cleaved and dephosphorylated pBR322 was ligated with genomic DNA. Ligated DNA sample was used to transform *E. coli* DH5α (Figure 5). The transformants were selected using starch agar plates. 10 transformant were grown separately in liquid medium containing 1.0% soluble starch as carbon source. Recombinant plasmid was isolated and digested with Hind III restriction endonuclease. In all cases, it showed presence of an insert DNA of variable sizes. Approximately, the sizes of the inserts were 2.8 to 3.2 kb. The Petri plates containing transformants were stained with I_2 and KI solution and showed clear starch hydrolyzing zone around the colony. The insert was with promoter and operator sites of the wild strain therefore helped in the expression of the cloned amy of AB-56. One of the transformants which showed maximum starch hydrolyzing zone was selected and was grown in potato starch. The extracellular amylase activity indicates positive cloning and expression of the amy of AB-56 in *E. coli* host. The transformant can be grown in potato starch medium for long time and after cellular lysis, the cell lysate can be used as single cell fertilizer.

DISCUSSION

The use of bio-fertilizer is pivotal to ensure sustainable soil fertility and to restore soil structure. Bio-fertilizers are non toxic and ecologically suitable. Therefore, agricultural scientists suggest using much amount of bio-fertilizers (Mohammadi and Sohrabi, 2012). However, living cells of bio-fertilizers incipiently compete for food and nutrition with the crop plants in an agricultural land (Sen, 2007). After the death and decomposition of a living cell, the cellular molecules will be freed and easily available to the crop plant as the source of nutrients. Due to this reason bio-fertilizers show a slow and antagonistic effect on cultivated plants (Sen, 2007) even though, many reports provided accelerated growth and yield by using various bio-fertilizers (Rao et al., 1983). To overcome this problem, the work focuses on the use of cellular molecules after the lyses of a cell as bio-fertilizer (Sen et al., 2013). The present study includes isolation of a

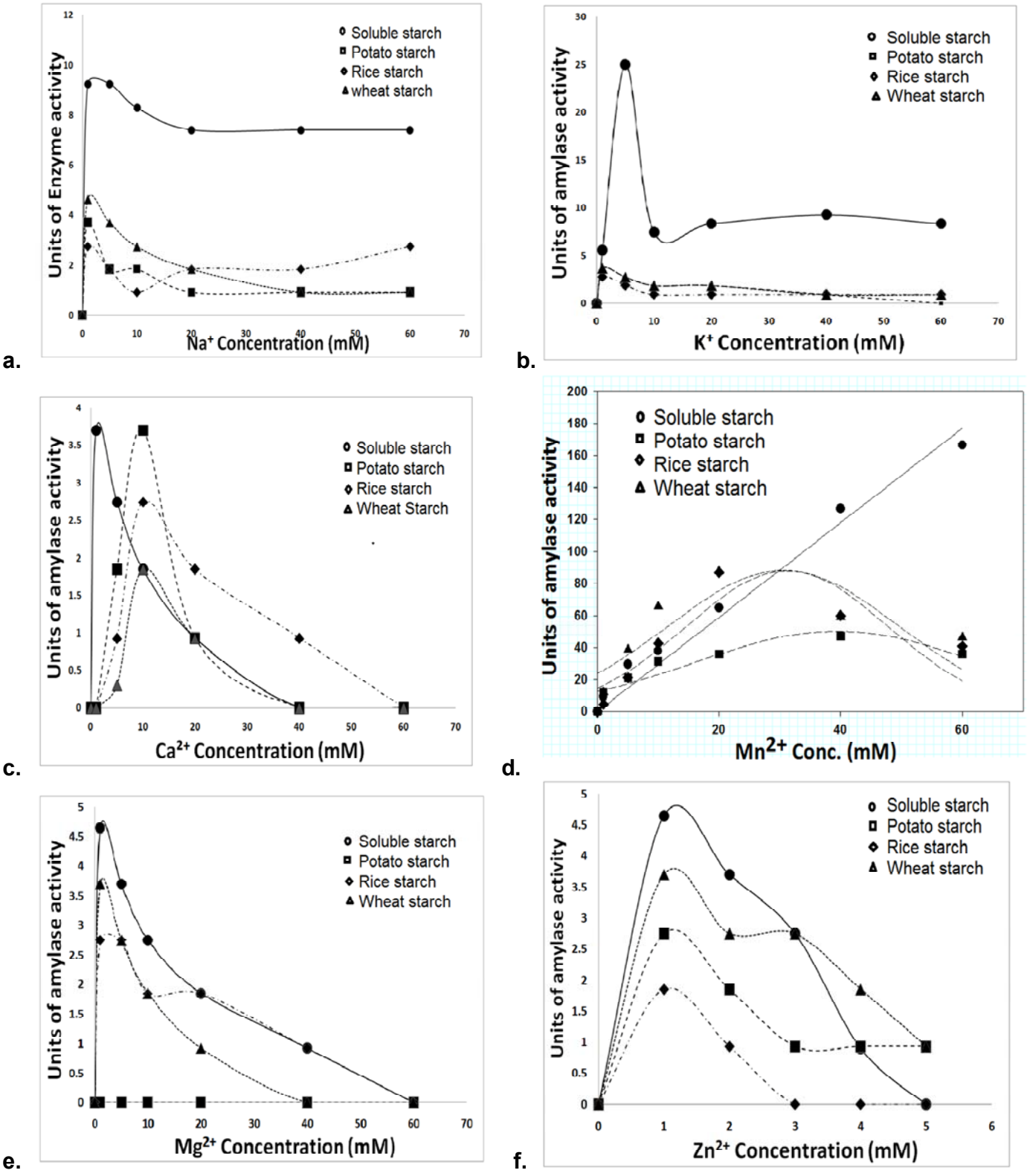

Figure 4. Effect of different metal ions on amylase activity. **a)** Effect of Na^+ concentration on amylase activity. Four various substrates were used separately in the assay. **b)** Effect of K^+ concentration on amylase activity. **c)** Effect of Ca^{2+} conc. on amylase activity. **d)** Effect of Mn^{2+} conc. on amylase activity. **e)** Effect of Mg^{2+} conc. on amylase activity. **f)** Effect of Zn^{2+} conc. on amylase activity. Other parameters in all the assays were remained same as described in Materials and methods.

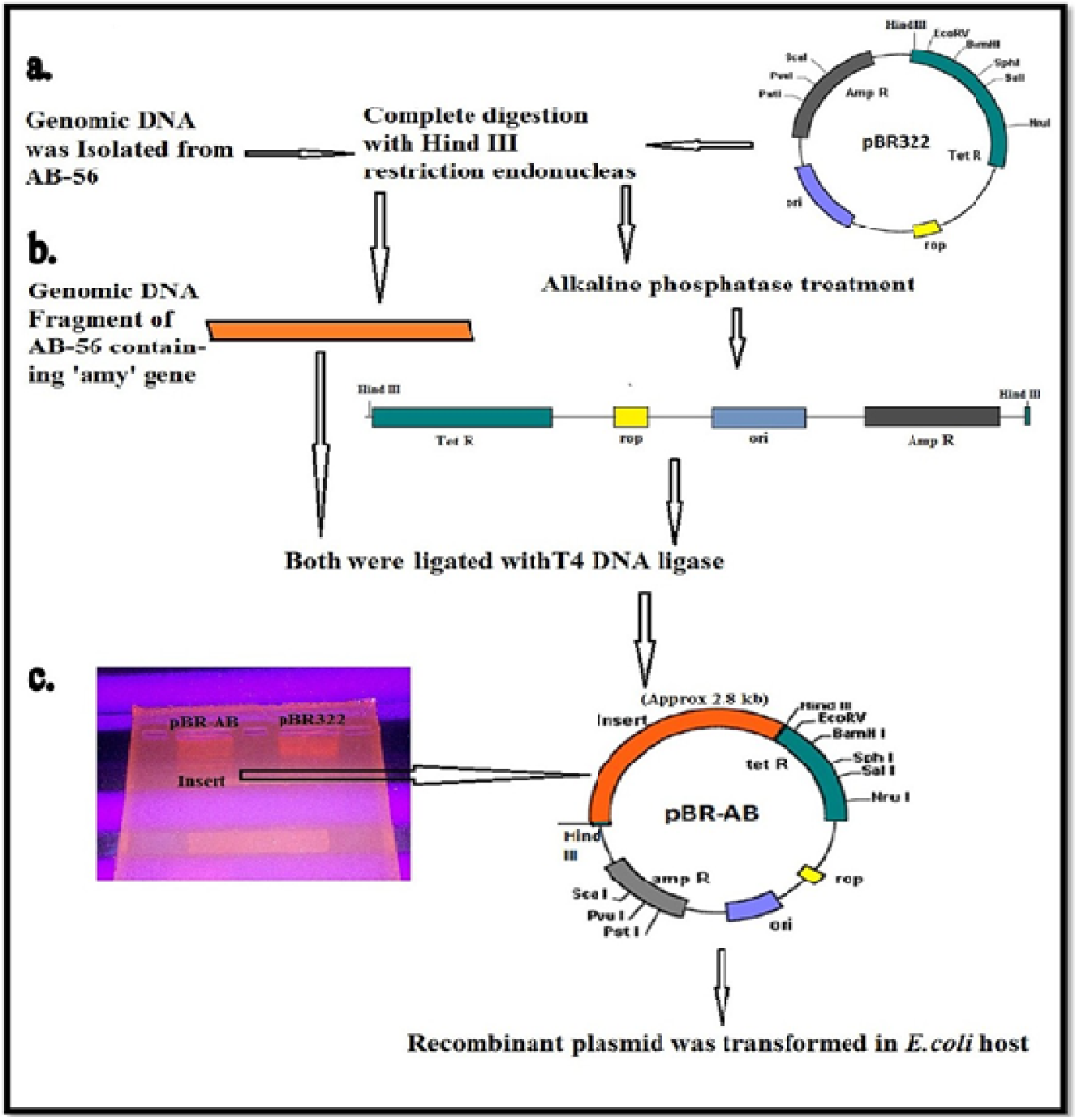

Figure 5. Strategy of cloning of 'amy' gene of AB-56. **a)** Genomic DNA and vector pBR322 isolated, purified and cleaved with Hind III; pBR322 was dephosphorylated. **b)** These DNAs were ligated with T4 DNA ligase. Ligated DNA was used to transform DH5α competent cells. **c)** Recombinant vector DNA was isolated and cleaved with Hind III. Figure shows the construct and the agarose gel containing the insert.

bacterium that uses potato starch as carbon source for its growth. Undefined starches are readily available with low cost in comparison to the more expensive industrially prepared starches (Nwagu and Okolo, 2011) and native starch sources significantly stimulated enzyme production (Nguyen et al., 2000). Therefore, the present study utilized potato starch as a suitable carbon source which can minimize the production cost (Kidd and Pemberton, 2002). On the basis of different biochemical tests, the organism was partially characterized as *Bacillus* sp. Impor-

tantly, starch, mannitol and carboxymethylcellulose induce extracellular amylase production of the organism whereas very low amount of intracellular amylase production in presence of various carbon sources indicates that amylase of the organism is mainly extracellular. The optimization of growth condition using different parameters like pH, temperature, potato starch concentration were carried out to get maximum growth of the organism. The organism grows above 50.0°C, therefore, it can be categorized as a thermo-tolerant bacterium. The analysis of amylase activity on different parameters showed some interesting results. Many reports support that the fungal amylase is optimally active at low pH whereas bacterial amylase is active in neutral pH (Patel et al., 2005; Nwagu and Okolo, 2011). The enzyme produced by the organism is active at low pH which is in agreement with the work of Demirkan (2011). This is probably due to the structure of the functional group in the active site which changed in basic condition and cause deformation (Al-Qodah et al., 2007). When soluble starch was used as substrate, optimum temperature for enzyme activity was at 17.0°C. This may be due to the better accessibility of the substrate to the active site at low temperature (Amico et al., 2003). K_m and V_{max} values were determined which are almost similar with the data obtained from the double reciprocal plot of substrate concentration versus velocity of enzymatic reaction (Al-Qodah et al., 2007; Demirkan, 2011). The enzymatic activity was increased upto 10 fold in presence of Mn^{2+} (Patel et al., 2005; Al-Qodah et al., 2007; Demirkan, 2011). According to some investigators Ca^{2+} is the main activator of amylase (Heinen and Lauwers, 1976; Bush et al., 1989; Demirkan, 2011). But here it was different. The molecular cloning of amy gene results improved production of amylase.

There are several reports on amy gene cloning and expression using Hind III restriction endonuclease (Mielenz, 1983; Sen and Oriel, 1989a; Sen, 2006). By growing a transformant using potato starch in a fermenter, huge amount of biomass can be produced in a short period which can be used as bio-fertilizer. The long term incubation of the culture caused cellular lyses after that it was used as bio-fertilizer to cultivate rice in field condition (data was not shown). However, liquid bio-fertilizer is more acceptable for farmers as it has more advantages over carrier based bio-fertilizer and can be considered as advanced bio-fertilizer production technology (Sheraz Mahdi et al., 2010).

Conclusion

Use of bio-fertilizer to improve structure and texture of agricultural land is essential because cellular molecules are organic molecules and eco-friendly. Production and supply of water-soluble nutrients as readymade food has a logistic prospect regarding the future aspect of bio-fertilizer. Encouraging the development of low-cost indigenous bio-fertilizer production is the main theme of the work by using a recombinant bacterium of this kind.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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