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Isolation and Identification of an Amylase Producing Trace Tolerance Bacteria from Industrial Waste of Haldia, an Industrial Town of India

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Abstract: *The aim of this study was to isolate and identify trace tolerance bacteria which produce amylase. We isolated bacteria from industrial waste of Haldia (GPS location: 22.025 88.058), West Bengal, India. We identified one amylase producing bacteria which can survive in wide range of pH 3 to pH 11 and temperature of 04°C to 50°C. It can tolerate heavy metals like cobalt, chromium, zinc, lead etc.. Among the six metal ions (Cu^{2+}, Zn2+, Pb^{+} , K^{+} , Mg^{2+} and Co^{3+},) the bacteria show more resistance against Cobalt acetate and less sensitive against Magnesium sulphate. Molecular characterization indicate that it belongs to Aeromonas caviae* NK1.

Keywords: *Bacteria, Amylase, Heavy metal, Enzyme assay*

1. INTRODUCTION

Industrial effluent is a source of various kinds of microorganisms [1]. Microorganisms isolated from Industrial effluent show different types of ability like resistance to heavy metals, degradation of different kinds of biomolecules, petroleum byproducts etc. [2, 3, 4]. Many bacteria isolated from waste water is a source of industrial enzymes like protease, lipase, amylase etc. [5,6,7].

Amylase is an enzyme that catalyzes the hydrolysis of starch into sugars residue. Amylases can be characterized as alpha, beta, and gamma amylases. α-amylase is a protein enzyme that hydrolyses alpha bonds of large, alpha-linked polysaccharides, such as starch and glycogen, yielding glucose and maltose [8]. It is commercially important as widely used in brewery and other industries [9]. There are various reports on starch degrading microorganisms from different sources and respective amylase activity [10]. The microbial amylases meet industrial demands; a large number of them are available commercially; and, they have almost completely replaced chemical hydrolysis of starch in starch processing industry [11, 12]. In this study we have isolated and identified trace tolerance bacteria from industrial waste of a factory situated at Haldia, an industrial zone, Purba Medinipur, West Bengal. This strain of bacteria can produce amylase and characterized the bacteria by morphological, physical, biochemical basis. We further characterized this bacteria by sequencing of 16S rRNA of the bacterial strain to ensure the genus of the organism.

2. MATERIALS AND METHODS

Industrial effluents were collected from sewage where the effluents from different sources are being discharged at different time period of a factory situated at Haldia- an industrial town of West Bengal, India. The GPS location of Haldia is 22.025 88.058.

2.1 Isolation of Organism

The bacteria were isolated by serial dilution (10^{-4}) of the effluents with starch containing mineral medium and plated in starch containing agar plate [5]. The plates were incubated at 37˚C for 24 h. After incubation, single colonies which formed clear halos with potassium iodide and Iodine solution (Ki/I solution) were identified as starch utilizing strains. Selected single colonies were further purified by repeated streaking and transferring to starch containing agar plate.

2.2 Characterization of the Bacteria

2.2.1 Morphological Characterization

Morphological characterization of isolated bacteria were done under bright field microscopy. The detailed characterization was conducted through various staining procedures like gram staining, endospore staining etc. The bright field microscopy was conducted at 100X magnification on Zeiss Axiostar Plus microscope.

2.2.2 Antibiotic Sensitivity Test

Antibiotic sensitivity of the bacteria was detected with 12 different antibiotics. The different antibiotics used and the detailed procedure for sensitivity assay was followed as reported by A.R.Thakur [6]. LB agar plate was prepared. Bacterial inoculums of size 10^{-5} cfu/ml was spread on the plate and commercially available antibiotic disks from HiMedia were used for antibiotic sensitivity test by disks diffusion methods. The plates were incubated overnight at 37^{o}C and observed for zone of inhibition. The 12 different antibiotic disks were gentamycin (10 µg), neomycin (30 µg), cefotaxime (30µg), ceftazidime (30 µg), vancomycin (30 µg), ampicillin (10 µg), polymyxin B (100 units), trimethoprim (30 µg), tetracycline(30 µg), doxcycline (30µg), cloxacillin (10µg), and metronidazole (4µg).These sensitivity was determined based on the diameter of the zone of inhibition and evaluation was done according to Manufacturers instruction provided in the kits by HiMedia [5, 7].

2.2.3 Biochemical Characterization

Biochemical characterization were determined by optimizing pH, temperature, growth phage, enzymatic assay, substrate utilization, heavy metal tolerance, etc.

2.2.3.1. Optimum pH and temperature for the growth of the bacteria: To determine the optimum growth pH, 0.1% of inoculum from overnight grown bacteria was cultured into sterile 50 mL Luria Bertani (LB) broth of different pH ranging from 3-11 and incubated at 37°C. For determination of optimum growth temperature, 0.1% inoculation was provided into LB medium having pH 7 and overnight incubation was done at different temperatures (04-50ºC). In both cases the culture were incubated under shaking condition at 150 rpm and growth was measured in terms of OD at 600 nm in bio spectrometer, (Eppendorf).

2.2.3.2. Heavy metal Sensitivity test: Metal salts such as $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $PbNO_3$, $K_2Cr_2O_7$, $MgSO_4$ and cobalt acetate were used for this study. LB medium with different concentrations of heavy metal ions were inoculated with overnight grown culture. The concentrations range of heavy metal in the LB medium were: $CuSO_4.5H_2O$ (1 to 10 mM); $ZnSO_4.7H_2O$ (1 to 10 mM); $PbNo_3$ (1 to 10mM); $K_2Cr_2O_7$ (0 to 5 mM); $MgSO_4$ (1 to10mM); cobalt chloride (0 to 5 mM). The cultures were incubated for 24 hours at 37˚C with shaking at 150 rpm. Growth rates of the strains were determined by absorbance at wavelength of 600nm (OD600) with bio spectrometer, (Eppendorf). Each experiment was done in triplicate [7, 8].

2.2.3.3. Substrate utilization: The Carbon Minimal Salt medium was supplemented with different organic substrates namely fructose, D-glucose, lactose, sucrose, starch, cellulose, sorbitol, etc. for determination of growth in different substrate condition. Phenol red was used as indicator of bacterial growth.

2.2.3.4. Amino acid utilization: In absence of nitrogen source bacteria cannot grow. If bacteria have enzyme to utilize any amino acid as a nitrogen source, then bacteria can grow in protein deficient medium. In a medium containing 1% glucose, 0.5% NaCl, different amino acids were supplemented for testing amino acid (lysine, alanine, tyrosine, tryptophan, proline and aspartic acid etc.) utilization [9].

2.2.3.5. Biochemical characterization by Enzyme assay: Biochemical characterization were performed by the following enzymatic assays such as amylase, catalase, oxidase, and nitrate reductase. Commercially available readymade media were used for detecting the presence of enzymes.

Oxidase test: Isolated single colonies from plate were picked up using a tooth pick and were gently scratched on the oxidase disks (DD 018, HiMedia Laboratories Pvt. Limited). Oxidase disks acts as an electron donator to cytochrome oxidase. If the bacteria oxidize (the disk remove electrons), the disk will turn purple (observation noted within 60 secs), indicating a positive test.

Catalase test: One isolated single colony, 1% H_2O_2 (Merck chemicals) was dropped in a slide and the appearance of bubble formation indicate the presence of catalase enzyme. The enzyme breaks down hydrogen peroxide (H_2O_2) by releasing free oxygen. The evolution of gas causes bubbles and it is indicator of a positive test.

Nitrate reductase test: One isolated single colony 1% H_2SO_4 and 3% solution-3 (for 100 ml $ZnCl_2$ 2 gm, starch 0.4 gm, KI 0.2 gm) were dropped using pipettes. If the appearance is blue colors, it indicating the positive test.

Amylase test: Overnight cultures, was streaked on the 2% starch containing medium and incubated at 37˚C for overnight. Starch hydrolysis was determined by the addition of Gram's iodine solution in to the overnight incubated plate. As a result, full plate appearance was blue but organisms which produce amylase appear as colonies surrounded by a clear zone.

2.2.4 Molecular Characterization

DNA was isolated from bacteria by alkaline lysis methods [10]. PCR amplification of the 16S rDNA gene fragment was done using universal 16S rDNA primers. The sequence obtained was subjected to nucleotide BLAST. Phylogenetic analysis was done by neighbor joining method Liu et al., 2007 [13].

3. RESULTS AND DISCUSSIONS

3.1 Cultivation, Optimization of pH, Temperature, and Sensitivity Test of Carbon and Amino Acid Source, of the Isolated Bacteria

The isolated bacteria are Gram positive, cocci having amylase producing ability. The ability of the isolated bacteria to produce amylase was initially confirmed by the observation of zone of clearance around the culture colonies grown in starch agar plate when flooded with KI reagent (Fig.1). The growth pattern was recorded at OD600 indicated that the log phase start after 2 h but predominantly it is in stationary phase onwards 10 h (Fig.2). The isolated bacteria are survived at a wide range of pH 3 to11 and grow well in the range of temperature 4^0C to 50^0 C. The optimum pH for the growth of the isolated bacteria is 7 (Fig. 3) and temperature is 37^0 C (Fig. 4) in LB media containing 0.1% inoculums and incubated under shaking condition at 150 rpm. Table 1 reported the substrate utilization profile (carbon and amino acid source) of the isolated bacteria. The result indicate that without aspartic acid, tryptophan, alanine, tyrosine, the bacteria could not grow. Carbon sensitivity test result indicate that in presence of starch and dextrose as carbon source, the bacteria show maximum growth.

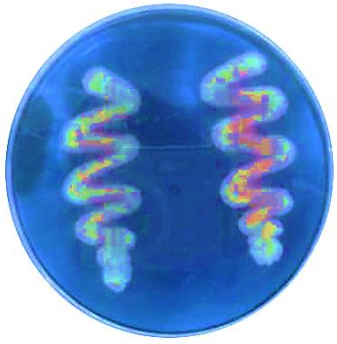

Figure1. *Test for Amylase activity of the isolated bacteria*

Table1. *Substrate utilization (Carbon & Nitrogen) profile of the isolated bacteria*

Fructose	+
Sucrose	+
Lactose	+
Starch	+ + +
Dextrose	+ ++
Cellulose	-
Sorbitol	+
Alanine	-
Lysine	+
Tyrosine	-
Tryptophen	-
Prolin	+
Aspartic acid	-

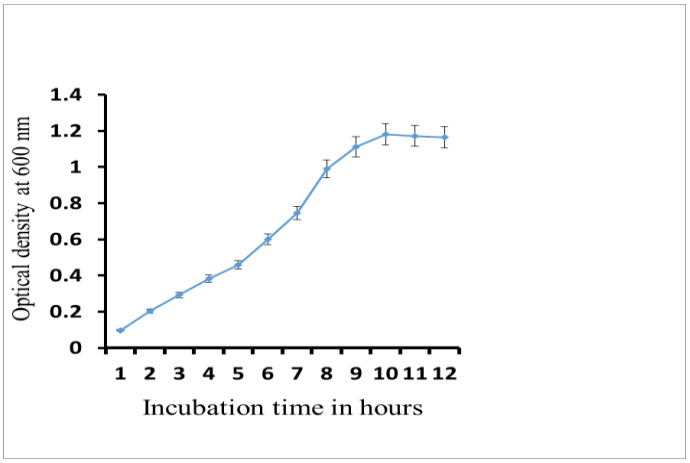

Figure2. *Optimum growth phase measured by OD at 600nm*

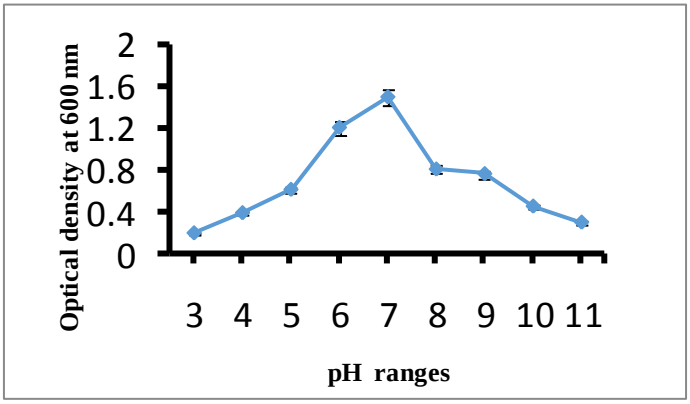

Figure3. *pH profile of the isolate*

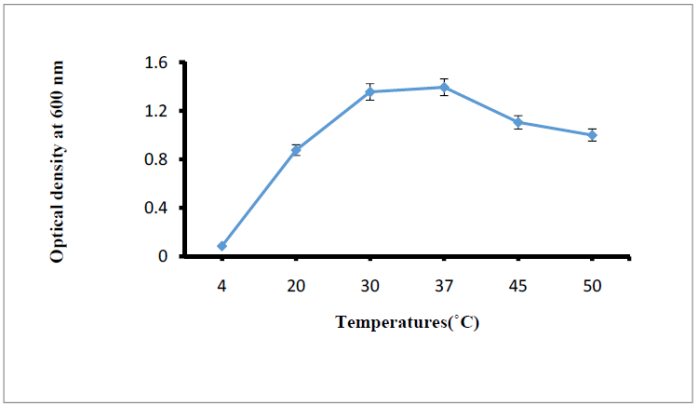

Figure4. *Temperature (˚C) profile of the isolate*

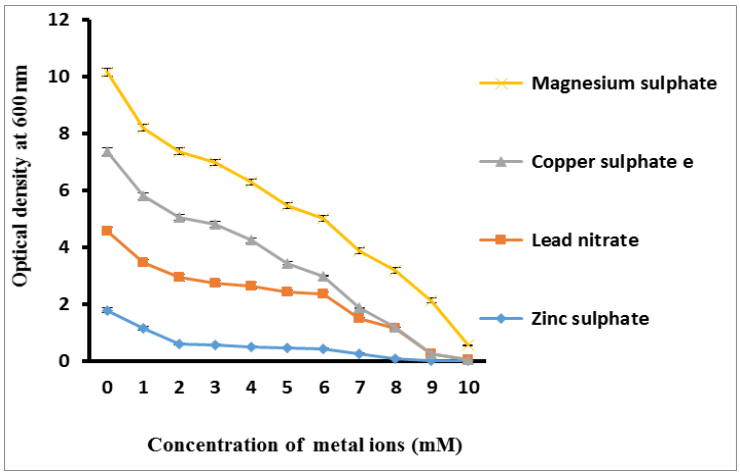

Figure5a. *Heavy metal sensitivity test*

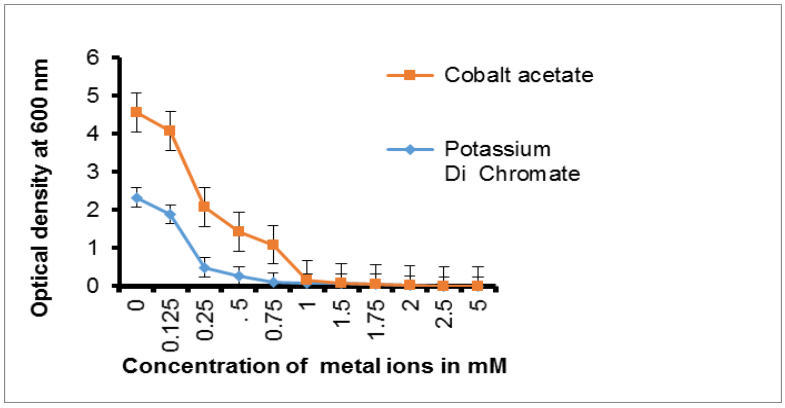

Figure5b. *Heavy metal sensitivity test*

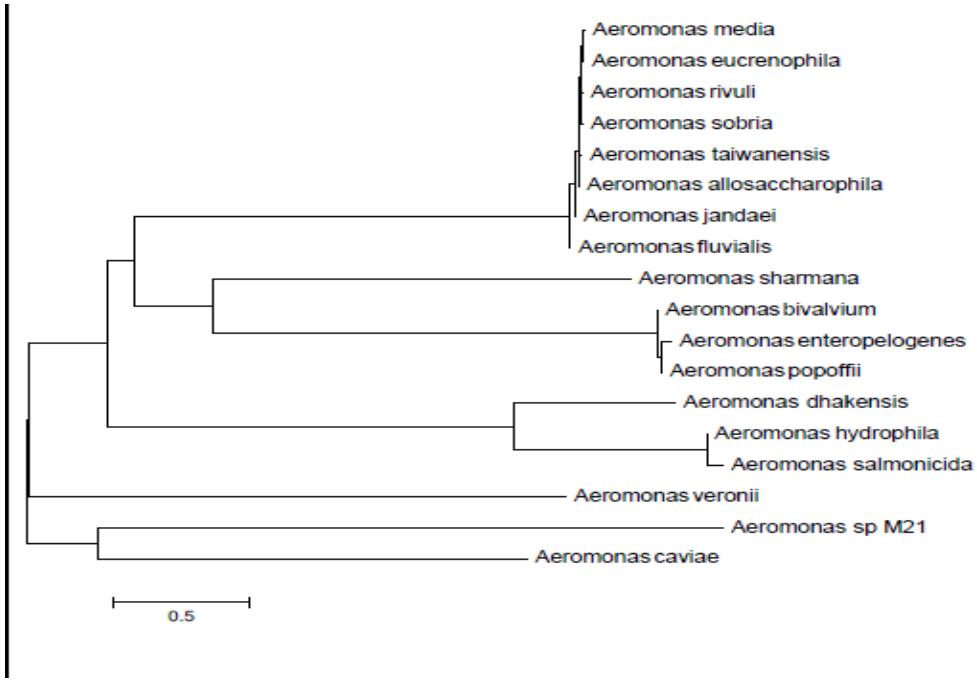

Figure6. *Phylogenetic tree of isolated Aeromonassp showing the relationship with other members of the genus Aeromonas using 16S rRNA sequence analysis by MEGA 6*

3.2 Antibiotic and Heavy Metal Sensitivity Test

Twelve antibiotics were used for the antibiotic sensitivity test of the isolated bacteria. Among them the bacteria show resistance against Vancomycin, Cloxacillin and the bacteria is sensitive against the remaining 9 antibiotics (Table 2). With respect to the heavy metal sensitivity test it was observed that

with increasing concentration of the heavy metal the growth of the isolated bacteria is decreased. Among the six metal ions the bacteria sow more resistance against Cobalt acetate and less sensitive against Magnesium sulphate (Fig. 5a and Fig. 5b).

Table2. *Antibiotic sensitivity profile of the isolated bacteria. (S) stands for sensitive, and (R) stands for resistance*

Name of the Antibiotics	Diameter of the zone (mm)	Remarks
Amphicilin (AAA) 10µg	17	S
Cefotaxime (CE) 30µg	25	S
Metronidazole (MT) 4µg	9	R
Vancomycin (VA) 30µg	9	R
Cloxacillin (CX) 10µg	10	R
Polymyxin B (PB) 100µg	13	S
Gentamycin (GEN) 10µg	19	S
Doxcycline (DO) 30µg	16	S
Neomycin (N) 30µg	18	S
Ceftazidime (CAZ) 30µg	30	S
Trimethoprim (TR) 30µg	21	S
Tetracycline (TE) 30µg	20	S

3.3 Biochemical Characterization

The biochemical characterization of the isolated bacteria was performed by oxidase test, catalase test, nitrate reductase test and amylase test (Table.3). In oxidase test when single colonies was added to the oxidase disks it was turned to purple color which indicate that the bacteria are oxidase positive. In catalase test when single colonies of the isolate was added with the 1% H_2O_2 and the formation of gas bubbles confirmed that the bacteria is catalase positive. On the other hand, the appearance of blue color when single colonies was added into the nitrate reductase disks indicated the isolate is nitrate reductase positive. The amylase test was confirmed by the appearance of clear zone around the colonies into the 2 % starch containing media plate (Fig.1).

Table3. *Biochemical characterization of the isolated bacteria by enzyme assay. (+) stands for positive results. (-) stands for negative results*

Name of the enzymes	Remarks
Amylase	+
oxidase	+
catalase	+
nitrate reductase	+

3.4 Identification of the Bacterial Isolate

With the help of morphological and biochemical analysis the isolated bacterial stain may be a member of genus *Aeromonas* and so identified as *Aeromonas sp*. We also study the 16S rDNA sequence and Phylogenetic analysis was done by neighbor joining method [13]. The result indicate that the stain has 99% similarity with *Aeromonas caviae*. Hence it is a new strain named as *Aeromonas caviae* NK1.

4. CONCLUSION

In conclusion the current result indicates that a bacterium called *Aeromonas caviae* NK1 was isolated from an industrial zone which can grow in a wide range of pH and temperature. The isolated bacterial strain produces amylase and oxidase, catalase and nitrate reductase positive. In presence of starch and dextrose as carbon source the isolated bacteria show maximum growth. Among the twelve antibiotics the bacteria show resistance against Vancomycin, Cloxacillin. With respect to the heavy metal sensitivity test it was observed that with increasing concentration of the heavy metal the growth of the isolated bacteria is decreased. Among the six metal ions the bacteria show more sensitive against Cobalt acetate and Magnesium sulphate. From Phylogenetic analysis the stain shows 99% similarity with *Aeromonas caviae*. So this heavy metal tolerance bacteria may be useful for amylase production. It can withstand different heavy metal stress.

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