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Characterization of amylolytic bacteria *Bacillus subtilis* LC4 isolated from Sita kund hot spring, Bihar, India

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Abstract
Microbial amylases share one quarter of industrial enzyme market. Their wide industrial application and ongoing demand has encouraged researchers to explore for newer and cheaper sources. The present study aims at isolation and characterization of amylase producing bacteria from water samples of Sita kund hot spring, Bihar, India. A total of 08 bacteria were isolated of which 04 were amylase positive. Among positive isolates, LC4 which showed maximum halo zone as well highest amylase production was selected for further study. Based on phenotypic and genotypic characterization, it was identified as *Bacillus subtilis* LC4 (Accession no: MH583045). Further effect of physical parameters on amylase production were optimized via one variable at a time approach under submerged fermentation condition. The strain showed maximum amylase production after 48 h of incubation, 7.0 pH and 45ºC. The results were encouraging and signifies hot spring as a sink of potent source of extracellular enzymes.

Keywords: amylolytic, Bacteria, Microbial, *Bacillus subtilis*

1. Introduction
In the era of global industrialization, replacement of harmful chemicals with enzymes is a forwarding step towards cleaner and greener technology. Amongst various enzymes, amylases share 25% of the world enzyme market [1], so are of special interest. They are starch degrading enzymes that hydrolytically cleave α -1, 4-glycosidic bond and α -1, 6-glycosidic bond in starch yielding glucose, maltose, maltotriose and oligosaccharide units [2]. They are commercially exploited in food, textile, detergent, leather, paper, pharmaceutical, distilling industries, waste management as well as clinical and analytical chemistry research areas [3, 4]. The extensive potentials of amylase to be used in broad range of industries have placed enormous stress on researchers to discover more efficient amylase sources. Microbes, plants and animals are the chief sources of amylase [5], where microbial amylases are preferred over other sources, because of their plasticity and vast availability [6]. Thermostable amylase are produced by a variety of *Bacillus* species, *viz*., *Bacillus* sp. I-3, *B. subtilis* JS-2004, *B. caldolyticus* DSM405, *B. licheniformis* ATCC 9945a, because of their thermophilic properties and high conversion rates [7], [8, 9]. Submerged fermentation is recommended for commercial production of industrially important enzymes, as it has better control over parameters like pH, temperature, etc., leading to high productivity of enzymes [10].
In the last decade, hot springs have been an important biotope of microorganism source of potentially robust enzymes because of their diverse and unusual and extreme nature thereby drawing attention of researchers towards hunt novel enzymes. Hot springs are niche, where geothermally heated water emerges out from the earth's crusts. A number of reports are on amylases and other industrially important enzymes from Indian hot springs [11, 12, 13, 14]. Fortunately, Bihar is bestowed with a number of hot spring located along the Rajgir-Munger metasedimentary belt of Munger, Nalanda and Gaya districts which attract a large number of tourists from different parts of the country as well as China, Japan, Tibet, etc., for pilgrimage. Sita kund is one such hot spring 4 miles east of Munger town making it the most visited hot spring. These sites could be rich repository of microbial diversity and the most desired enzymes, which have not been systematically traversed as per my knowledge. Keeping these facts in mind the present work aims at isolation and characterization of amylase producing bacteria from Sita kund hot spring.

2. Materials and methods
2.1 Sampling
Sampling was done from sampling sites *viz*., Sita kund located in Munger (25.38°N, 86.46°E) district of Bihar, India. Samples were collected at the depth of 20 cm in sterile bottles and under aseptic condition and processed immediately. Temperature and pH of sample were measured at the time of collection.

2.2 Isolation and screening of amylolytic bacteria
Bacteria were isolated from water sample of hot spring by serial dilution and streak plate methods. The aliquots (0.1 ml) were plated on Nutrient Agar (NA) medium [(w/v) 0.5% peptone; 0.3% beef extract; 0.5% NaCl; 1.5% agar, pH 7] and incubated at 50±2°C for 72 h. Isolated pure cultures were primarily screened for amylase activity by employing halo zone technique on starch agar plate.

2.3 Identification of amylolytic bacteria

Phenotypic characterization of the isolate was done by different tests referring to Bergey's Manual of Determinative Bacteriology and Agriculture handbook [15, 16]. For genotypic characterization, genomic DNA was extracted from the isolate using Chromous Genomic DNA isolation kit (RKT09). The amplification of 16S rRNA gene was carried out by using Thermal cycler (ABI 2720) in 100 µl reaction mixture containing 2.5 mM each of four dNTP, 10X PCR buffer, 3U of Taq DNA polymerase, 10 ng template DNA and 400 ng each of primer (F) 5'-AGA GTR TGA TCM TYG CTW AC-3' primer (R) 5'-CGY TAM CTT WTT ACG RCT-3'. The amplification programme was set as initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 2 min and a final extension at 72°C for 5 min. The sequencing was performed according to the manufacturer's protocol using Big Dye Terminator Cycle Sequencing Kit (v3.1, Applied Biosystems) and analyzed in an Applied Biosystems Analyzer. The sequence of 16S rDNA (1.189 kb) was aligned by using the BLASTN program to identify the most similar sequence in the database and phylogenetic tree was constructed bioinformatics software MEGA 7.0 after alignment of the sequences with the Clustal W software [17].

2.4 Enzyme Production by submerged fermentation

Enzyme production was carried out by submerged fermentation (SmF) process in 250 ml Erlenmeyer flasks containing 60 ml of basal medium [(w/v) 0.6% peptone; 0.05% $MgSO4.7H_2O$; 0.005% $CaCl_2$, 0.2% $(NH4)_2SO_4$, 0.05% KCl; 1% soluble starch; pH 7], inoculated with 1% (approximately $2x10^6$ CFU/ml) overnight (24 h) grown culture, centrifuged at 10,000× g for 10 min at 4°C and supernatants were used for estimation of enzyme activity.

2.5 Enzyme Assay

Amylase assay was done by incubating reaction mixture containing 1 ml enzyme and 1 ml soluble starch (1% in 0.1M phosphate buffer, pH 6.5) at 50°C for 10 min. The reaction was stopped by adding 3 ml of 3, 5-dinitrosalicylic acid reagent (DNS). The absorbance was measured at 540 nm using double beam UV/VIS spectrophotometer (Systronics, 119). The reducing sugar released was measured by the method of [18]. One enzyme unit (U/ml) is equivalent to the amount of enzyme needed to release 1µ mole of reducing sugar.

2.6 Optimization of physical culture conditions for amylase production

Optimization of production medium was done by OVAT approach, where one independent variable is studied at a time, keeping the other parameters constant. Effect of various physical parameters, *viz.*, incubation period, pH and temperature were investigated for optimum production of amylase.

2.6.1 Effect of incubation time

It was studied by incubating bacteria in basal production medium for different time periods i.e., 24, 48, 72, 96 and 120 h at initial temperature of 50°C and pH 7.0. Amylase production was recorded after an interval of 24 h.

2.6.1 Effect of pH

Effect of pH on enzyme production was studied at varying pH i.e, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 of production media. 1N HCl and 1N NaOH were used for modifying the pH of the production medium.

2.6.2 Effect of temperature

Effect of temperature on amylase production was studied by incubating bacteria in production media at different temperatures, i.e., 30, 35, 40, 45, 50, 55, 60, 65 and 70°C, keeping above optimized conditions under consideration.

2.7 Statistical analysis

Effect of each parameter was studied in triplicate and graphically represented as the mean ±SE (n=3) using MS Excel.

3. Results and Discussion

3.1 Sampling

Water samples from Sita Kund hot springs was collected by decapping and recapping the bottles at the depth of 20 cm to avoid aerial contamination. The temperature of water sample was slightly high i.e., 50°C, while he pH was slightly acidic i.e., 6.3. Previous workers have reported that the thermal springs of Rajgir area was slightly acidic (pH: 5.3-5.8), whereas cold springs of Munger area are near neutral to slightly alkaline (pH: 7.1-8.4) in nature [19]. It is opined that the temperature and pH of different hot spring varies and that is governed by the depth from which geothermal water is emerging out, physical conditions, chemical composition, cultural and social activities associated with it.

3.2 Isolation and screening of amylolytic bacteria

Based on colony morphology, a total of 08 different bacterial colonies were isolated on NA plates at 50 ºC and pH 7.0 and were designated as LC1, LC2, LC3,… LC8**.** 04 isolates (LC1, LC4, LC5, LC7) out of 08, were found to be amylase positive (Table 1). Further they were quantified for amylase production after 48 h of incubation, which clearly reflected that halo zone diameter of bacterial colony was in direct proportion to that of the amount of amylase produced. In this quantification, LC4 showed highest production (15.09 U/ml). Thus, on the basis of prominent halo zone by LC4 (Fig 1) and consequently highest amylase production, it was selected subjected to identification.

Fig 1: Clear halo zone on starch nutrient agar plate by isolate LC4

Table 1: Sampling, isolation and screening of amylase producing bacteria

Temperature*	pH*	Bacterial isolates	Amylase positive isolates			
			Total No.	Nomenclature	Halo zone (cm)*	Amylase Production (U/ml)
50º C	6.3	08	04	LC1	0.45	6.43
				LC4	0.9	15.09
				LC5	0.54	8.02
				LC6	0.32	6.22

*****Values are means of triplicates

3.3 Identification of amylolytic bacteria

Phenotypic identification of selected isolates i.e., LC4 was done on the basis of morphological and biochemical parameters. Microscopic observations revealed that the isolate was gram positive, motile rods and endospore formers (Table 2). Upon biochemical test gave positive reaction for catalase production, citrate utilization and VP test, while negative for indole production, oxidase reaction, MR test, H_2S production and urea hydrolysis. The isolate hydrolyzed starch, casein and gelatin (Table 2). On the basis of phenotypic characteristics, isolates were assigned to the genus *Bacillus.*

Table 2: Morphological and Biochemical characterization of isolates

Morphological Parameters	
Colony morphology	Regular, flat, Pale yellow
Cell size (µm)	2.23×0.89
Motility	+
Gram Test	+
Shape	Rod
Endospores	Sub-terminal
Biochemical Parameters	
Catalase test	+
Oxidase test	-
Indole test	-
Methyl Red (MR) test	-
Voges Proskauer (VP) test	+
Citrate utilization	+
Oxidation/Fermentation (O/F)test	F
Cytochrome oxidase test	+
Casein hydrolysis	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Urea hydrolysis	-
Nitrate reduction	+
H_2S production	-

\+ - Positive reaction
\- - No Reaction
F - Fermentation Reaction
W - Weak Reaction

Identification of isolate on molecular basis revealed 99% identity with the type strain *Bacillus subtilis*. The phylogenetic tree based on bacterial 16S rDNA sequences and closest reference strains, downloaded from database, showed a significant relationship with *Bacillus subtilis*, hence identified as *Bacillus subtilis* LC4 (Fig 2). The sequence of identified strain was deposited at the NCBI Genbank under accession no. (Accession no: MH583045).

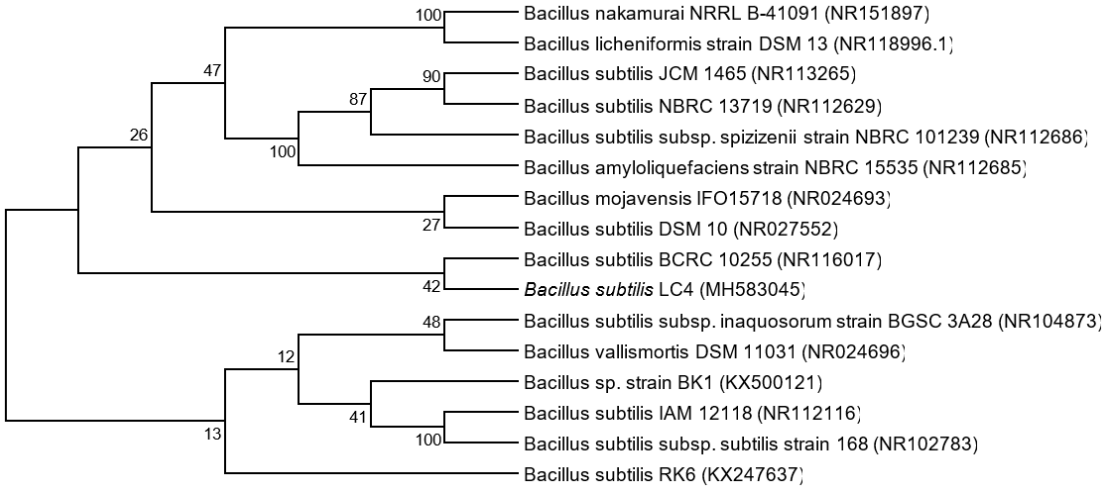

Fig 2: Phylogenetic tree showing genetic relationship of *Bacillus subtilis* with taxonomically similar strains, species and genus based on 16S rDNA sequences. Bootstrap values based on 1000 replicates are shown next to the branches

3.4 Optimization of physical culture conditions for amylase production

3.4.1 Effect of incubation period on amylase production

In the present work, selected strains were incubated for different time interval in basal medium at 50°C and pH 7.0 to determine optimum incubation time for amylase production. An incubation period of 48 h was found to be most apt for maximum amylase production i.e., of 24.64 U/ml (Fig 3). Upon further incubation, enzyme production declined (Fig 3). As pointed out by earlier workers extracellular amylase production is associated with growth of microbes [20, 21]. During exponential phase of growth, availability of enough nutrients and metabolites positively affect microbial growth and enzyme concentration. Optimum amylase production at 48 h of incubation is supported by previous studies in *B. subtilis* MTCC121 [22] and *B. subtilis* ATCC6633 [23]. Several reasons has been enumerated to explain gradual reduction in enzyme production *viz*., depletion of nutrients in culture medium, misbalance between available nutrients and biomass,) cultures entering death phase, degradation of enzyme in presence of other components in the fermentation medium, sugar accumulation over a critical concentration in the medium, etc. [24, 25].

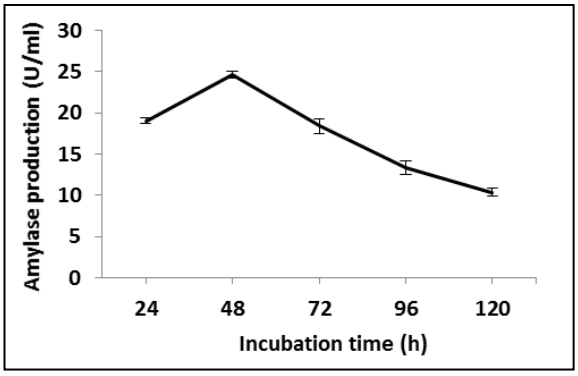

Fig 3: Effect of Incubation time on amylase production

3.4.2 Effect of pH on amylase production

PH of production medium is a critical factor for synthesis, activity and stability of any enzyme, as it plays a crucial role in enzyme secretion and also induces morphological changes in organisms [26, 27], so its optimization is essential in this study. In the present study, pH range of 5.0 to 9.0 was studied to determine the optimum pH amylase production, where pH 7.0 appeared as most suitable for *B. subtilis* LC4 (28.12 U/ml) when incubated optimum incubation time (Fig. 4). At pH of 8.0 and 9.0 amylase production was quite low suggesting that the strains prefer acidic to neutral pH i.e., of 6.0-7.0 for amylase production. The most interesting observation made in this work is that pH of sampling site (approximately 6.3), from where *B. subtilis* LC4 was isolated, as well as the optimum *in vitro* pH for bacterial growth and enzyme production (7.0) were similar. This supports the view that the habitat's condition coincides with inhabiting organism's growth optima. It has been remarked that fungi requires slightly acidic pH, whereas bacteria need neutral pH for optimum growth and production of enzymes [28]. It has been reported that optimum pH range for bacterial growth and enzyme secretion is 6.0 to 7.0 [26], while, highest amylase production by *Bacillus amyloliquefaciens was* obtained at pH 7.0 [29]. Their findings are similar to our observation, where neutral pH appeared most suitable for maximum secretion of amylase.

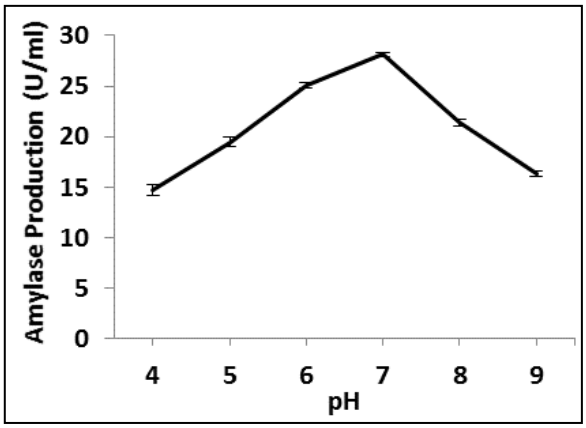

Fig 4: Effect of pH on amylase production

3.4.3 Effect of temperature on enzyme production

Like pH, microorganisms are also profoundly affected by temperature of their habitat as it influences their growth and metabolite secretion. Since, at low temperature growth rate is slow, less enzyme is produced. However, as the temperature rises up to optimal limit, due to enhancement in growth rate, more biomass is produced leading to higher enzyme secretion. In order to determine the optimum temperature for amylase production, were grown in the temperature range of 30- 70°C (Fig. 5). In this study, 45°C was found to be optimum for amylase production in *B. subtilis* LC4 (44.21 U/ml) which were almost similar to the temperatures of their habitat. Incubation above and below their respective optimal temperatures resulted in reduced bacterial growth and enzyme production. Present results show the organism's inclination towards thermophilic nature. A wide range of temperature (35-80°C) has been reported for optimum growth and α-amylase production in bacteria [30, 31]. Maximum amylase production at 37°C and 40°C has also been documented in *Bacillus amyloliquefaciens* [29] and *Bacillus subtilis* ATCC 6633 [23], respectively.

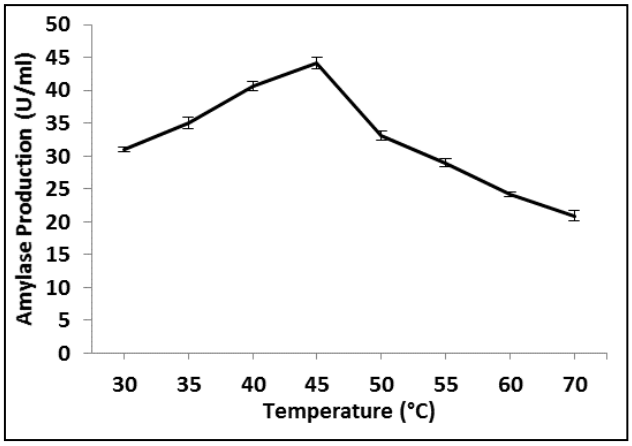

Fig 5: Effect of temperature on amylase production

4. Conclusion

Each strain shows variation in optimum growth condition in terms of physical and chemical parameters, reflecting physiological needs of organism are variable and governed by their genotype and environmental influences. The present work revealed that *B. subtilis* LC4 showed growth from 30°C upto 70°C with optimum temperature for amylase production at 45°C thus reflecting its thermotolerant nature. Optimum pH for amylase production was 7.0, while its habitat pH was 6.3. It can be concluded that, *B. subtilis* LC4 is a potential producer of extracellular amylase. The findings holds key of further research for scaling up fermentation by optimization of other fermentation condition and characterization of purification of amylase.

4. Acknowledgement

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