Diauxic Growth Pattern in Thermophilic *Bacillus* spp with Respect to Production of Thermostable Amylase

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Abstract. Thermophilc bacteria are emerging as source of biotechnological applications in recent years. The present study is focused on the growth patterns of thermophilic bacteria, isolated from the Soldhar hot spring (Indian Himalaya), with respect to amylase production. The morphologically and physiologically different bacterial isolates viz. GBPI_30, GBPI_31 and GBPI_35, identified using 16S rDNA gene sequencing, showed maximum similarity with *Bacillus licheniformis*, *B. subtilis* and *B. licheniformis*. These isolates were able to grow in wide temperature 25 to 85 ℃ (optimum 55 ℃) as well as wide pH (5-14) range. While the bacterial isolates followed normal growth pattern in Tryptone Yeast extract broth, they exhibited diauxic growth pattern with respect to amylase production in starch broth. Maximum amylase production was recorded during the stationary phase of growth at different temperatures i.e. 65 ℃, 35 ℃ and 55 ℃ for GBPI_30, GBPI_31, and GBPI_35, respectively. Amylases of the bacterial isolates showed different temperature optima for enzyme activity i.e. 40 ℃, 60 ℃ and 50 ℃, while pH optima was recorded similar for all the three isolates. Amylase production in variable temperature conditions by thermophilic *Bacillus* spp. would be advantageous for their survival in natural habitat along with the associated biotechnological applications.

Key words: Thermophiles, *Bacillus*, growth curve, amylase, hot spring, Indian Himalayan region

1 Introduction

Microorganisms that grow in extreme environmental conditions are referred as extremophiles [1]. Extreme environmental conditions may include physical (e.g. temperature, radiation, pressure) and geochemical conditions (e.g. desiccation, salinity, pH, oxygen requirement, redox potential). Among these, temperature is one of the most important environmental factors that control the activities and evolution of the organisms [2], including microorganisms. Thermophilic proteins are studied due to their potential applications in biotechnology [3]. In nature, thermal preferences range from hyperthermophilic to psychrophilic [4]. The microorganisms that grow well in the temperature range between 45-113 ℃ are referred as thermophilic microorganisms [5]. Thermophiles have been isolated from various extreme environmental habitat including oil reservoirs, deep aquifers, continental hot spring, shallow marine hydrothermal system and deep sea vent [6]. Hot springs serve as one of the richest niches for the growth of thermophilic microorganisms and characterized by hydrothermal systems that constantly release geothermally heated water above earth surface. These hot springs are also characterized by low and/or fluctuating nutrient conditions, therefore, microbiota in such environments rely on several survival strategies to maintain their existence [7]. For instance, bacteria consume the simplest carbon sources (preferably glucose) followed by complex ones. When multiple carbohydrates are present in the nearby environment, bacteria result in biphasic or diauxic growth patterns. The mechanism behind this strategy is known as carbon catabolite repression [8]. Such features require attention while conducting the studies to understand the ecological resilience dependant biotechnological applications associated with the extremophilic microorganisms.

Thermophilic microorganisms produce several intracellular as well as extracellular enzymes that have importance in their survival under extreme environment stress [9,10]. These enzymes possess unusual

characters regarding their stability and activity at higher temperatures and, therefore, have several commercial applications [11]. Amylases are important hydrolytic enzymes that catalyze the hydrolysis of starch into diverse products that include dextrins and smaller polymers of glucose [12]. These enzymes have importance in biotechnological industries and rank an important position in the global enzyme market (25 % to 33 %) [13]. Commercially, a variety of bacteria and fungi including *Bacillus subtilis, B. licheniformis, Micrococcus halobius, Aspergillus oryzae* and *A. niger* are used for amylase production [14]. Thermostable amylases have advantages in industries, such as decreasing contamination risk, increasing diffusion rates, increased solubility of starch and decreased viscosity, being resistant to denaturing agents, solutions and proteolytic enzymes [15,16].

Low as well as high temperature environments in Indian Himalayan region (IHR) are receiving attention with respect to the colonization of extremophiles possessing unique characteristics, such as, tolerance to wide range of pH and temperature along with their biotechnological applications including the production of bioactive compounds [17,18]. Thermophilic microorganisms reported from various hot springs include the phototrophic bacteria (*Cynobacteria*, purple and green bacteria), eubacteria (*Bacillus, Clostridium, Thiobacillus, Desulfotomaculum, Thermus, Spirochetes* and *actinobacteria*) and Archaea (*Pyrococcus, Thermococcus, Thermoplasma, Sulfolobus* and *Methanogens*) [10,19,20]. The hot springs namely Soldhar and Ringigad, located in the Uttarakhand state of Himalaya, initially came in picture for its general microbial diversity, applications and conservation [21-24]. Later, following conventional and molecular techniques, these hot springs were studied for distinct groups of microorganisms including unculturable diversity and their resilience to high temperature [10,19,25,28]. The focus of the present study is on the growth patterns of the three selected thermophillic *Bacillus* isolates with respect to the production of amylase along a temperature range.

2 Material and Methods

2.1 Bacterial Isolates, Characterization and Scanning Electron Microscopy (SEM)

Three thermophilic bacteria, originally isolated from the soil sediments of the Soldhar hot spring, were procured from the Microbial Culture Collection established in the Microbiology Lab of GBPNIHESD. Bacteria were characterized based on morphology, microscopy and several biochemical tests including carbohydrate fermentation, IMViC, urease, nitrate reduction, catalase, H_2S production and hydrolysis of casein and lipids using standard procedures.

SEM was also performed for bacterial structure. Bacterial isolates were incubated for 24 h in Tryptone Yeast extract broth. Following incubation, bacterial cells were extracted by centrifugation at 10,000 rpm for 10 min at 4 ℃ and supernatant was removed. Pellet was then fixed with 2.5 % glutaraldehyde (prepared in 0.05 M phosphate buffer) for 4 h at 4 ℃. After fixation, samples were washed with phosphate buffer thrice. After washing with phosphate buffer, samples were further washed with distilled water thrice. A thin film of sample was placed on the 2 mm thin cover slip and dehydrated using light source. Samples were coated with gold by using JFC 1600 gold coater and viewed under JSM 6610LV (JEOL Model) microscope.

Tolerance to different physiological conditions of temperatures, pH and salt was also determined by growing bacteria at different temperatures on TY agar or by observing growth of bacteria in TY broth of varying conditions of pH and salt as described in Pandey et al. [10].

2.2 Genotypic Identification and Phylogenetic Tree

Molecular identification of the bacterial isolates was done following 16S rRNA gene sequencing using universal eubacterial primers (Courtesy: Microbial Culture Collection, NCCS, Pune, Maharashtra, India). The BLAST analysis was then performed using NCBI database and phylogenetic tree was reconstructed using MEGA v6 [29]. The type cultures and their nucleotide sequences are deposited in Microbial Culture Collection, National Centre for Cell Science, Pune, India and NCBI, respectively.

2.3 Growth Pattern in TY and Starch Broth

Growth pattern of the bacterial isolates was studied at four different temperatures, i.e. 35 ℃, 45 ℃, 55 ℃ and 65 ℃ in two media broth to examine the growth behavior. TY broth was considered as general purpose medium while starch broth (composition in g/L: corn starch 5g, yeast extract 5g, $(NH_4)_2SO_4$ 2.5g, $MgSO_4.7H_2O$ 0.2g, KH_2PO_4 3g, and $CaCl_2.2H_2O$ 0.25g) as selective medium for amylase production. Growth curves were prepared by inoculating 12 h grown bacterial cultures in 100 ml respective media in 250 ml Erlenmeyer flasks and measuring OD_{600} using a Ultrospec 2100 pro UV/Vis spectrophotometer after every 6 h interval up to 48 h.

2.4 Amylase Production at Different Temperatures

The bacterial isolates were screened for amylase production on starch agar (Hi media). Point inoculation of bacterial culture was done following incubation of the agar plates at 55 ℃ for 24 h. Following incubation, plates were flooded with Gram's iodine and observed for formation of clear zone around the bacterial colony.

Amylase production by the bacterial cultures was carried out in 250 ml conical flask containing 100 ml starch broth after inoculation with 100 µl (0.1 % v/v) of 12 h pre-grown culture and incubated at four different temperatures, i.e. 35 ℃, 45 ℃, 55 ℃ and 65 ℃ for 48 h. For determination of enzyme produced, 2 ml culture broth was collected aseptically after every 6 h and centrifuged at 8000 rpm for 10 min. Cell free supernatant was used for the determination of amylase activity. All the experiments were performed in triplicates.

2.5 Determination of Enzyme Activity

Quantitative determination of amylase activity was carried out using dinitrosalicylic acid (DNSA) method [30]. 300 µl of crude enzyme was mixed with 500 µl of 0.2 M phosphate buffer (pH 6 for GBPI_30 and GBPI_31 and pH 5 for GBPI_35). 200 µl of 1 % (w/v) of corn starch was added to reaction mixture and incubated at room temperature for 2 min. After incubation, the reaction was stopped by adding 1 ml of DNSA reagent and all the tubes were kept in a boiling water bath for 10 min. After cooling, the change in colour was measured at 540 nm against substrate blank as well as the enzyme blank. One unit of amylase activity was defined as the amount of enzyme required to release 1.0 µg of glucose per min under specified assay condition.

2.6 Ammonium Sulfate Precipitation

All the three bacterial cultures were grown in starch broth for obtaining maximum amylase to carry out its partial purification. After production, bacterial culture was centrifuged and cell free supernatant was subjected to ammonium sulfate precipitation at 4 ℃ (80 % saturation). The precipitate was dissolved in minimal amount of sodium phosphate buffer (0.5 M) of active pH and dialyzed overnight against same buffer at 4 ℃.

2.7 SDS PAGE and Zymogram Analysis

The dialyzed enzyme preparation was subjected to SDS-PAGE (12.5 %) analysis under non-reducing conditions. After electrophoresis, the gel was kept in 2.5 % Triton X-100 in desired buffer for 1h to remove SDS [31]. After washing, gel was incubated in same buffer containing 1 % starch for 1h at 55 °C. Gel was finally stained with iodine solution (0.15 % I_2 in 1.5 % KI). Enzyme activity in gel as pale yellow bands was visible against dark blue background. A parallel gel with standard molecular weight marker was stained with Coomasie brilliant blue R250 (CBB) to determine approximate molecular weight of amylases.

2.8 Enzyme Activity and Stability

Effect of temperature on amylase activity was investigated after incubating enzyme reaction mixture at different temperatures from 10-100 ℃ in sodium phosphate buffer (100 mM; pH 5 for GBPI_30 and pH 6 for GBPI_31 and GBPI_35). Thermal stability of partially purified amylase was determined by

incubating the enzyme at different temperatures for one h. Residual activity was then measured by assaying the enzyme reaction at optimum temperature in sodium phosphate buffer of respective pH as described earlier.

Optimal pH for amylase activity was determined by performing enzyme assay in buffers of different pH ranging from 3-11 (for GBPI_30) and 4-11 (GBPI_31 and GBPI_35) at optimum temperature. Similarly, for pH stability, enzyme was incubated in different buffers for one h and residual activity was measured after performing standard enzyme assay at optimum pH. Buffers (100 mM) used for different pH include citrate buffer (pH 3), citrate phosphate buffer (pH 4-5), sodium phosphate buffer (6-7), Tris-Cl (pH 8-9) and glycine-NaOH buffer (pH 10-11).

2.9 Statistical Analysis

Variation in enzyme activities was estimated by one way ANOVA followed by post hoc Tukey's HSD test to find significant differences between enzyme productions at different temperatures. Mean and standard errors of three replicates was calculated using Microsoft Excel 2007 software.

3 Results

3.1 Bacterial Characteristics and Phylogenetic Tree

All three *Bacillus* isolates showed moderate growth on TY agar medium after 24h of incubation at 55 ℃. Distinct single colonies were visible after 12h of incubation only, which were off white to cream colored. Colony morphology, microscopy and SEM microscopy of three isolates is presented in Fig 1. All the isolates observed as Gram positive rods under microscope and possessed a terminal spore. GBPI_30 could grow in temperature ranging from 20-70 ℃ while GBPI_31 and GBPI_35 could grow from 20-80 ℃ while optimum growth of all the isolates was achieved at 55 ℃. All the isolates showed pH tolerance from 5-14 with optimum growth at pH 7.

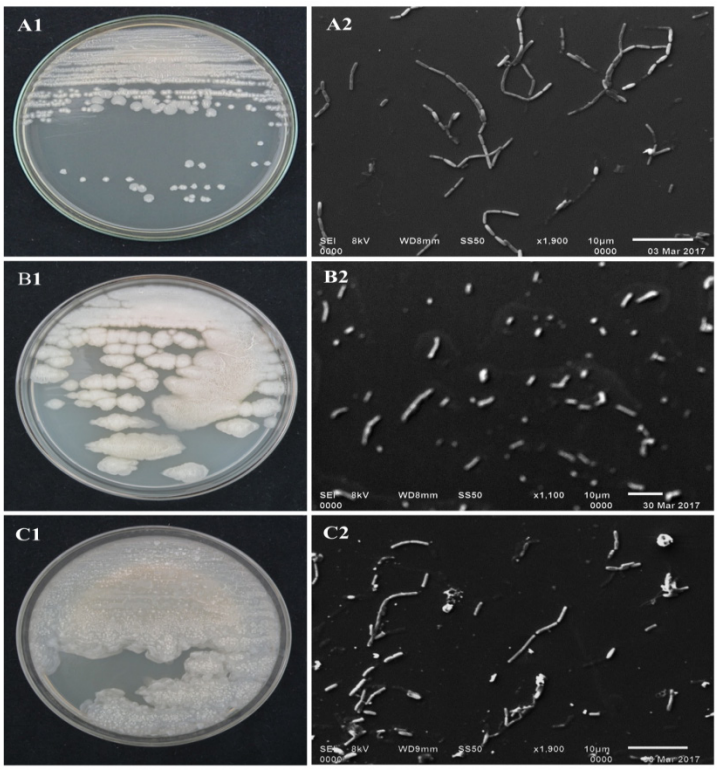

Figure 1. Colony morphology and SEM images of bacterial isolates at optimum temperature. A1&A2, B1&B2 and C1&C2 are morphology and microscopic images of GBPI_30, GBPI_31 and GBPI_35, respectively.

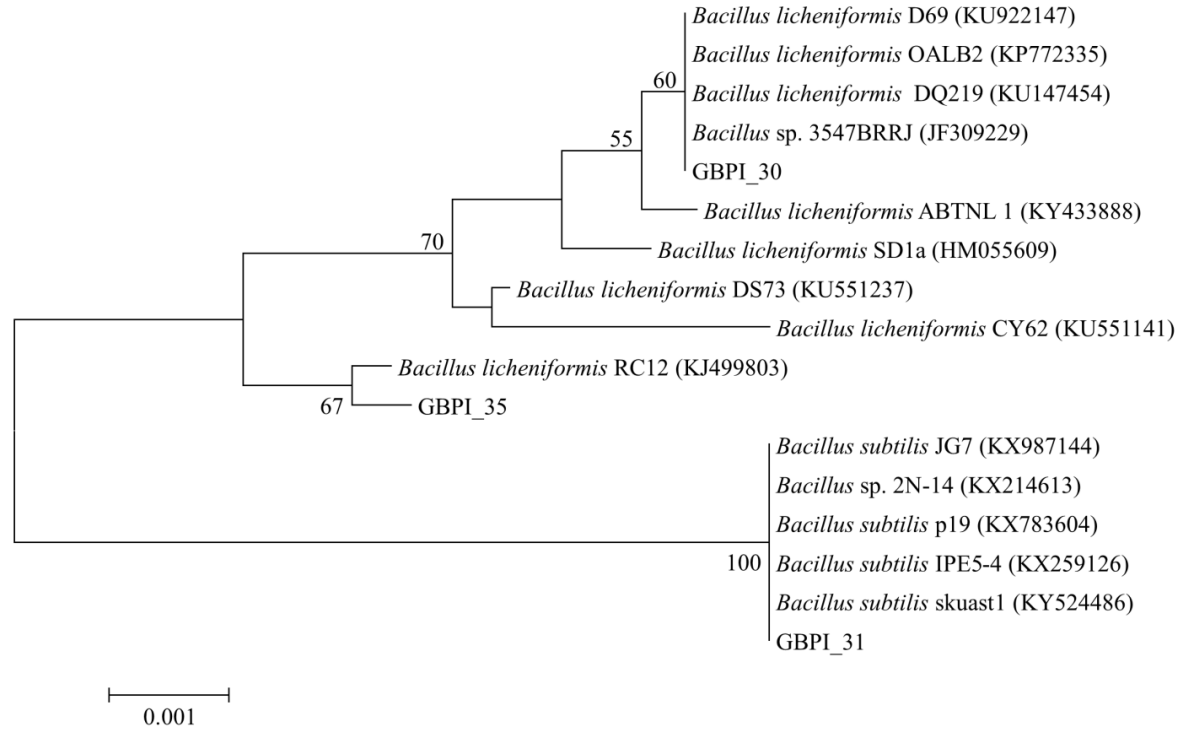

Figure 2. 16S rRNA gene sequence based phylogenetic tree showing the relationship of bacterial isolates GBPI_30, GBPI_31 and GBPI_35 with the other closely related members of the genus *Bacillus*. The tree was reconstructed using the Neighbour Joining method, the model used was the Kimura two-parameter model and phylogenetic confidence was inferred using 1000 replicates for bootstrapping.

Morphological, microscopic and biochemical characters of three bacterial isolates are presented in Table 1. On the basis of these characters, the bacterial isolates were assigned to the genus *Bacillus*. The bacterial isolates i.e. GBPI_30, GBPI_31 and GBPI_35 were given species level after BLAST analysis of PCR amplified 16S rRNA gene sequence that showed maximum similarity with *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus licheniformis*, respectively. The bacterial isolates GBPI_30 and GBPI_35, identified as *B. licheniformis* varied in their morphological, physiological and growth patterns. The phylogenetic tree of three *Bacillus* spp reconstructed by Neighbor Joining method is shown in Fig. 2.

Table 1 Morphological and biochemical characteristics of three *Bacillus* isolates and their identification

S. No.	Characters	GBPI_30	GBPI_31	GBPI_35
1	*Colony Morphology*	Off-white, smooth convex-round colony (dia 1.0-2.0 mm)	Grayish-white, irregular, rough colony (dia 4.5-5.0 mm)	Off- white, irregular, watery colony (dia 1.0-1.8 mm)
2	*Microscopy*	Gram +ve, long chains; single terminal oval spore; motile	Gram +ve, mostly single; oval spore arranged terminally at both ends; motile	Gram +ve, mostly diplobacilli; oval single terminal spore; motile
3	*Biochemical tests*	Gelatin, nitrate, methyl red and citrate utilization positive	Gelatin, catalase, nitrate, methyl red and citrate utilization positive	Gelatin, nitrate, indole, methyl red, VP and citrate utilization positive
4	*Carbohydrate utilization*	Galactose, fructose, sucrose and maltose positive	Fructose, arabinose, sucrose, maltose and sorbitol positive	Fructose, arabinose, sucrose, maltose and sorbitol positive

5	*Extracellular enzymes*	Lipase and protease positive	Lipase and protease positive	Lipase and protease positive
6	*Identification (% similarity)*	*Bacillus licheniformis* (99 %)	*Bacillus subtilis* (100 %)	*Bacillus licheniformis* (99 %)
7	*Culture Accession numbers*	MCC2912	MCC2908	MCC2909

3.2 Growth Pattern in TY and Starch Broth

Growth pattern in TY and starch broth along with amylase production was recorded for all the three isolates at different temperatures. The bacterial isolates, in general, showed normal growth at their optimum as well as suboptimal temperature in TY broth. Contrary to this, an erratic pattern of bacterial growth was observed in starch broth. The effect of different temperature on the growth of three thermophilic bacterial isolates in TY and Starch broth is shown in Fig. 3.

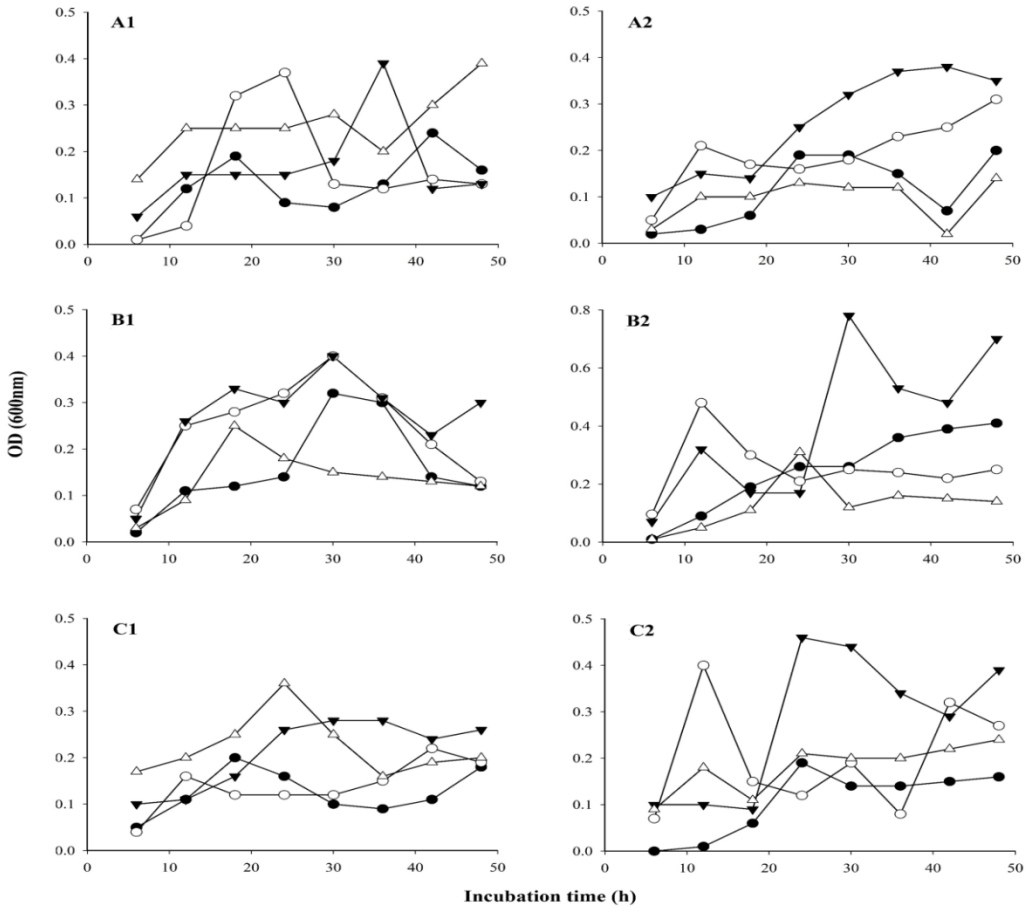

Figure 3. Growth curves of bacteria in TY and Starch broth at 35 °C (•), 45 °C (o), 55 °C (▲) and 65 °C (Δ). A1&A2, B1&B2 and C1&C2 are growth curves of GBPI_30, GBPI_31 and GBPI_35 in TY and Starch broth, respectively.

At 55 ℃, GBPI_30 showed typical bacterial growth in TY broth. The growth patterns at other temperatures showed variation from normal growth; with decrease/increase in cell density at later stages of the growth. The maximum biomass was recorded after 36 h of incubation at 55 ℃ in TY broth. In starch broth, biphasic (diauxic) growth was observed at all the temperatures from 35 to 65 ℃. Maximum cell biomass was achieved at 42 h of incubation in starch broth (Fig. 3A1&3A2).

Slow increase in cell biomass of GBPI_31 in TY broth was observed from 6 h of incubation that declined after 30 h at all the temperatures except at 65 ℃ where decrease in bacterial growth was observed just after 18 h of incubation. Diauxic growth pattern of growth along with amylase production was also recorded in case of GBPI_31. At 55 ℃, maximum growth was observed first at 12 h and later at 30 h of incubation. Similar growth pattern was recorded at 45 ℃ as well (Fig. 3B1&3B2).

In case of GBPI_35, maximum biomass was recorded at 65 ℃ after 24 h incubation in TY broth. In starch broth, two maxima of cell biomass were recorded indicating diauxic growth pattern of bacteria similar to other bacterial isolates. The maximum biomass in starch broth was recorded at 55 ℃ after 24 h of incubation (Fig. 3C1&3C2).

3.3 Amylase Production at Different Temperatures

The bacterial isolates showed amylase production in qualitative screening at different temperatures (Fig. 4). The effect of temperature on production of amylase enzyme by three bacterial isolates was estimated in starch broth in static conditions. The bacterial isolates were found to be active producer of amylase enzyme in temperature range from 35 to 65 ℃. Amylase production started from 6 h of incubation at all temperatures while the maximum activity varied with respect to the bacterial isolate and temperature (Fig. 5).

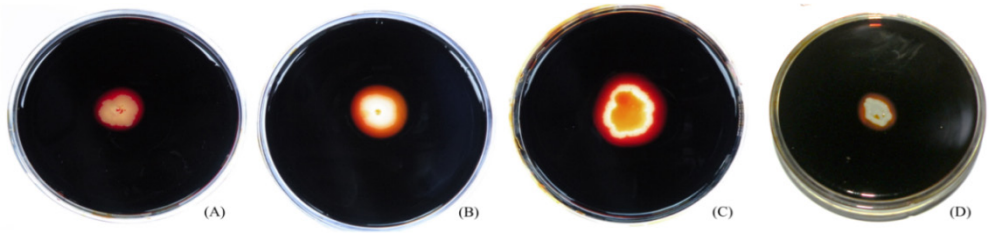

Figure 4. Qualitative assay for amylase production by GBPI_31 at different temperatures. (A) 35 °C (B) 45 °C (C) 55 °C and (D) 65 °C.

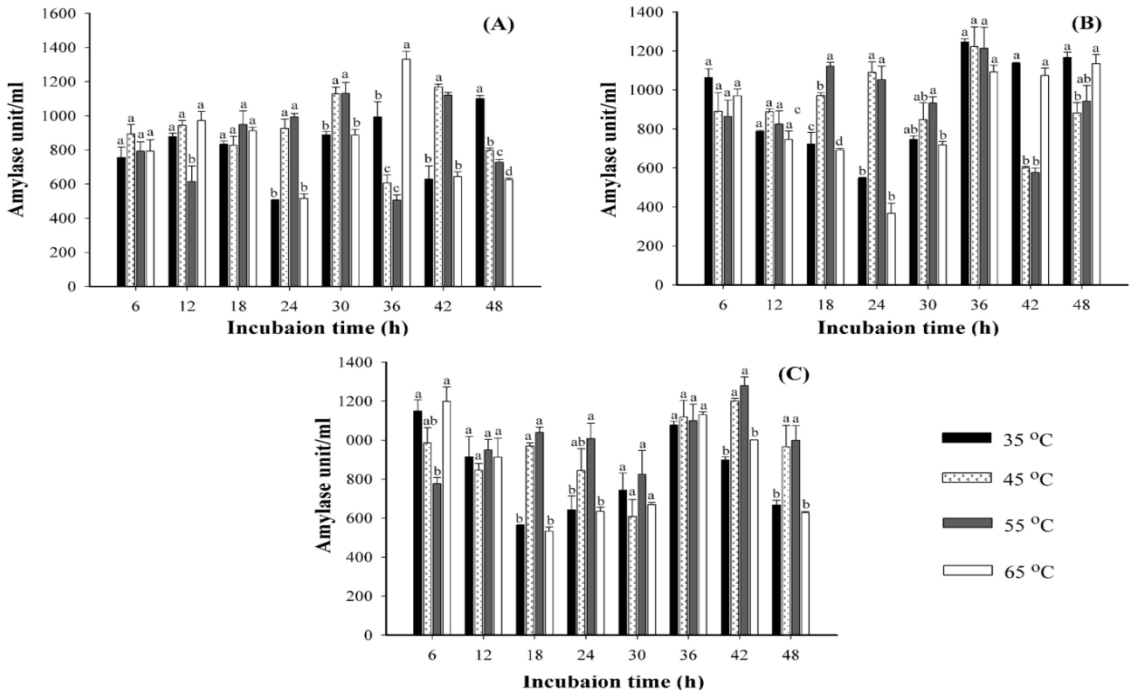

Figure 5. Amylase production by three bacterial isolates at different temperatures. (a) GBPI_30, (b) GBPI_31 and (c) GBPI_35. Different letter in a bar group indicates significant difference as measured by Tukey's HSD following one way ANOVA. Error bar = standard error (n=3).

In case of GBPI_30, maximum amylase production (1331.35 U/ml) was recorded after 36 h of incubation at 65 ℃ that was significantly different ($p<0.05$) from the enzyme production at other temperatures. Maximum enzyme production at 45 ℃ and 55 ℃ was observed after 30 h as well as 42 h of incubation that was not statistically different ($p<0.05$) (Fig. 5a).

No statistically significant difference ($p<0.05$) was observed in case of maximum enzyme production after 36 h of incubation by GBPI_31 at all the four temperature conditions. Minimum production of enzyme was recorded at 65 ℃ after 30 h of incubation which increased on further incubation up to 48 h (Fig. 5b).

GBPI_35 produced maximum amylase (1279.37 U/ml) at its optimum growth temperature of 55 ℃ after 42 h of incubation; production not being significantly different ($p<0.05$) from the production obtained at 45 ℃. Very high enzyme activity was recorded after 6 h of incubation that after further incubation showed production of enzyme in erratic manner (Fig. 5c).

3.4 SDS-PAGE and Zymogram Studies

The enzymes produced by three *Bacillus* spp showed very high molecular weight after non reducing SDS-PAGE following zymogram analysis (Fig. 6). Out of three isolates, GBPI_30 and GBPI_35 possessed amylase of similar molecular weight (>200 kDa) while the amylase of GBPI_31(~110 kDa) differed in its molecular weight. The high molecular weight of amylase might have observed due to multimeric form of the protein produced by all three *Bacillus* isolates.

Figure 6. SDS-PAGE followed by zymogram analysis of enzyme preparations from three bacterial isolates. Lane 1-GBPI_30, 2-GBPI_31 and 3-GBPI_35

3.5 Enzyme Activity and Stability

GBPI_30 showed maximum enzyme activity at 40 ℃ with maximum stability recorded at 50 ℃ (Fig. 7A). Sharp decrease in the activity and stability was recorded below and above these indicated temperatures. Optimum pH recorded for amylase activity as well as maximum stability produced by GBPI_30 was recorded at pH 5 (Fig. 7B). Sudden decline in enzyme activity was recorded beyond this pH. In case of isolate GBPI_31, 60 ℃ was the optimum temperature for amylase activity with maximum stability recorded at 40 ℃ (Fig. 7C). On the other side, pH 5 was the optimum activity as well as stability pH for maximum enzyme functioning (Fig. 7D). Isolate GBPI_35, unlike other two isolates showed optimum activity at 50 ℃ while enzyme retained two optima for stability i.e. 40 ℃ and 60 ℃, respectively (Fig. 7E). Similar to other isolates, GBPI_35 also possessed maximum activity and stability at pH 5 (Fig. 7F).

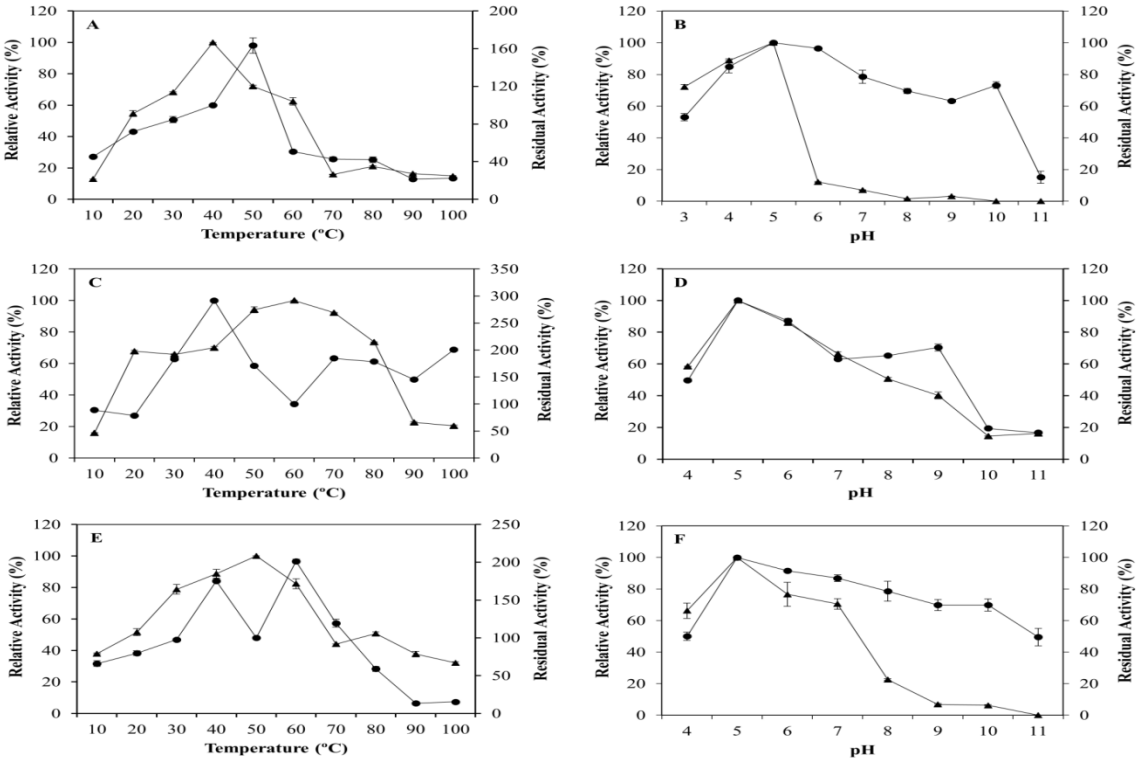

Figure 7. Effect of temperature and pH on activity (▲) and stability (●) of amylases produced by three *Bacillus* strains. (A&B) GBPI_30, (C&D) GBPI_31, (E&F) GBPI_35.

4 Discussion

Microbial diversity of various hot springs around the world along with their utilization for production of several enzymes of industrial importance have been reported in several studies [32-34]. The present study emphasizes the growth of three *Bacillus* spp at different temperatures with simultaneous production of amylase enzyme. All the three bacteria were able to survive at wide temperature range (20 ℃ to 80 ℃). This property of isolates to tolerate wide temperature range could be due to their environment of origin, i.e. soil sediment of hot springs. Unlike hot spring water, temperature of soil sediments might fluctuate due to variation in surrounding temperature (subzero to mesophilic temperature) throughout the year. This fluctuation might have provided bacterial isolates the ability to tolerate such wide temperature variation. For microorganisms to grow in such extreme conditions, their ability to survive in wide temperature range is the most important strategy [35]. Tolerance to wide pH range (5-14) by these bacteria could be another mechanism to grow in extreme environments, such as hot springs. The pH tolerance by microorganisms growing in extreme environments has recently been reviewed by Dhakar and Pandey [17]. Tolerance to such extreme conditions of growth by microorganisms from various hot springs around the world has also been reported in several studies [10,36,37].

The thermophilic bacteria, characterized on the basis of their morphological, cultural and biochemical traits in this study were confirmed as species of *Bacillus* on the basis of molecular identification. Various strains of thermophilic *Bacillus* spp have been reported from extreme temperature environments of hot springs. Thermophilic bacteria are well identified for the production of various thermostable enzymes with their biotechnological applications [38,39] as well as ecological adaptations [25,40]. In this study, growth of bacteria along with role of amylase in high temperature survival, considering enzyme production an ecological resilience feature has been taken in to account.

In general, a normal growth pattern was followed by all the three *Bacillus* isolates in TY broth at all the temperatures with few exceptions. This indicates towards their adaptability through ecological residence at such high temperatures without disturbing their growth properties. This is supported by the

previously discussed fact that the tolerance to temperature is the most important strategy for survival under extreme conditions. In contrast to TY broth, in general, all the bacterial isolates showed diauxic growth at all the temperatures in starch broth that is characterized by first utilization of simple substrate followed by the complex one. Starch being the complex substrate caused bacteria to show diauxic growth while yeast extract being the readily used substrate providing all necessary growth factors to achieve first growth maxima. There are evidence of diauxic growth in microorganisms during utilization of complex sugar (e.g. starch and maltose) leading to amylase production [41]. Prakash et al. [42] explained this behavior probably due to the reflected inhibition during the stationary phase and catabolic repression by the glucose released from maltose and maltotriose as the stationary phase continues. This has been proposed as a possible way for microorganisms to conserve energy.

Higher amylase production was achieved at early growth or late stationary phase of the thermophilic *Bacillus* spp. In case of GBPI_31 and GBPI_35, the amylase production was observed in suboptimal temperature conditions as well as in early growth stages while at optimal growth temperature it reached maximum in stationary phase of growth. In contrast, GBPI_30 produced maximum lipase at suboptimal temperature (65 ℃) in late phase of growth. Several studies have reported different *Bacillus* species, including *B. cereus* and *B. subtilis*, with maximum amylase production during early or late stationary phase of growth [43], *B. amyloliquefaciens* [44] and *B. licheniformis* [12,45]. In the present study, amylase production in early growth stages might be due to the utilization of yeast extract along with starch as substrate. Yeast extract in production medium is known to stimulate amylase production [46]. Many researchers have studied the correlation between α-amylase secretions with temperature that depends on the type of organism and culture conditions. Incubation temperature affects all the physiological activities in a living cell and it is an important environmental factor to control the growth, microbial activities and the normal functioning of enzymes. Several enzymes control the nutritional requirement of the cell and subsequently its composition [47]. The correlations developed, in the present study, between cell biomass and amylase production at different temperatures did not show any correlation (data not shown) between these parameters. This indicates toward no relationship between the bacterial enzyme production and the growth.

While determining the molecular weights of amylases using SDS-PAGE under non-reducing conditions it was found that the enzymes showed either aggregation or enzyme production as multimeric proteins and therefore showed higher molecular weight than expected (>200 kDa). In general, the molecular weight of amylases of microbial source lies between 40-70 kDa [48]. Several species of *Bacillus* are reported to produce amylase with molecular weight lying between 55 and 60 kDa [12,49,50]. Molecular weight of amylase produced by GBPI_31 was found to be ~110 kDa which could be suggested due to extracellular secretion of amylase as dimeric protein. Another reason for high molecular weight of amylases from thermophilic bacteria, in the present study, could be attributed to the secretion of protein with higher carbohydrate content. Such features could be responsible for masking the effect of adverse conditions and, therefore, help displaying ecological resilience by bacteria.

An interesting feature of the current study is that all the *Bacillus* spp showed optimum activity at different temperatures from 40 ℃ to 60 ℃. Despite differences in their temperature optima, all the three isolates showed similar pH optima of pH 5, while the stability decreased at both low and higher pH. Similar pH optima from 5-7 of amylase from different *Bacillus* strains has been reported in various studies [12,48]. Thermostable enzymes from three different isolates could be suitable for several catalysis processes in multiple industries which require variation in temperatures along with the stability of enzymes in wide range.

The present study gives an insight towards the survival of microbial life under the extreme temperature. It demonstrates the role of amylase in ecological resilience possessed by the thermobacilli along with the preliminary information for the novel biotechnological products. This information can be utilized further for the better understanding of remarkable physiological behavior, such as tolerance to extreme pH and temperature, in thermophiles. The enzyme production and its stability at high temperature is likely to be important in the industrial processes along with the specialized protein structure contributing to the adaptation studies.

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Conflict of interest statement

The authors declare no conflicts of interest.

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