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REGULAR ARTICLE

PRODUCTION OF INDUSTRIALLY IMPORTANT ENZYMES BY THERMOBACILLI ISOLATED FROM HOT SPRINGS OF INDIA

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ABSTRACT

Enzymes from thermophilic bacteria have received great attention for their potential applications in various industrial sectors. The present study deals with the production of five thermozymes (amylase, lipase, xylanase, protease and cellulase) from 10 thermophilic bacterial species, originally isolated from two hot springs namely Soldhar and Ringigad in Uttarakhand Himalaya, India. The bacterial isolate GBPI_25 produced maximum amylase (1217.86 U/ml) at 45 °C and 5 pH, GBPI 3 produced maximum lipase (22.59 U/ml) at 65 °C and 9 pH, GBPI_25 produced maximum xylanase (98.07 U/ml) at45 °C and 9 pH, GBPI_35 produced maximum protease (16.66 U/ml) at 55 °C and 9 pH, and GBPI 4 produced maximum cellulose (108.68 U/ml) at 45 °C and 5 pH. Crude enzyme preparations showed thermal and pH activities at broad temperature and pH range between 10-100 °C and 3-11 pH, respectively, with different temperature and pH optima. Amylase, xylanase and cellulase showed maximum activity at 50 °C while lipase and protease showed higher activity at 40 and 60 °C, respectively. Enzyme activity at wide temperature range-cellulase and protease from 10-100 °C, amylase and xylanasefrom10-90 °C, and lipase activity from 10-80 °C were the remarkable records from this study. Similarly, pH range for amylase and lipase activity was recorded from 4-11, for xylanase from 3-9, and for protease and cellulase from 3-10. All the thermozymes showed maximum stability at 40 °C and pH 5 except cellulase that showed higher stability at40 °C and neutral pH.

Keywords: Hot springs, Thermobacilli, Thermozymes, Enzyme activity, Enzyme stability

INTRODUCTION

Enzymes are biological catalysts that involve in numerous metabolic and biochemical reactions. They are widely applicable in biotechnological industries such as paper, pulp, food, textile, beverage, pharmaceutical and cosmetics. The demand of industrial enzymes has been projected to be US$ 7,100 million in 2018 with a yearly increase at 8% progression [1]. Enzymes from microorganisms have several advantages like lower cost, higher product recovery, ease for genetic manipulation, regular availability and rapid in growth over plant and animal derived enzymes. Out of nearly 4000 known enzymes, about 200 microbial enzymes are applicable at commercial levelwhile20 enzymes are produced at industrial scale [2]. Enzymes from the microbial sources are known for better-quality and extracted from a range of microorganisms including bacteria, actinobacteria and fungi and used for commercial applications [3].

Extremophilic microorganisms are capable for the production of extremozymes which have unusual properties like salt allocation, thermostability and adaptability [4]. These enzymes are responsible for the survival of extremophiles in stressed environmental conditions and also support various biotechnological applications. Extremozymes like amylase, lipase, xylanase, cellulase and protease have wide applications in many biotechnological processes. These enzymes have been used since many years in paper, pulp, food and pharmaceutical industries [5].

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Indian Himalayan region (IHR), due to its different topographic, geographic and climatic conditions, is increasingly getting attention for its so far untapped microbial diversity. Hot springs, namely Soldhar and Ringigad located in Chamoli district of Uttarakhand state in IHR, have been a focus for understanding the basic and applied aspects of the microbial diversity of thermophiles. These thermophilic organisms are important in view of their bioprospecting including their diversity, applications and conservation [6-11].

Several environmental factors influence the production of large amount of enzymes. Media composition, temperature and pH are among the important factors that have pivotal impact on economy and viability of the production of enzymes, therefore, standardization of these factors is important [12]. In this background, the present study deals with 10 selected thermophilic bacteria (species of *Bacillus*, *Geobacillus* and *Paenibacillus*) with particular reference to the production of 5 industrially important enzymes along with the activity and stability at different physiological conditions i.e. temperature, pH and incubation time.

MATERIALS AND METHODS

Bacterial isolates and characterization

Qualitative screening

10 thermophilic bacteria (species of *Bacillus, Geobacillus* and *Paenibacillus*), originally isolated from the sediments of two hot springs namely Soldhar and Ringigad, were taken from the Microbial Culture Collection established in Microbiology Lab of the Institute (GBPNIHESD). The bacterial cultures were identified on the basis of their phenotypic and genotypic characters as GBPI 3-*Geobacillus stearothermophilus*; GBPI 4-*G. kaustophilus*; GBPI13-*G. stearothermophilus*; GBPI_16-*G. stearothermophilus*; GBPI_22-*Bacillus licheniformis*; GBPI_25-*B. tequilensis*; GBPI_30-*Paenibacillus ehimensis*; GBPI_31-*P. ehimensis*; GBPI_35-*B. licheniformis*; GBPI_37-*B. sonorensis* [10,13-15]. General description of these thermophilic bacteria are presented in table 1.

The bacterial species were examined for the production of 5 extracellular enzymes namely amylase, lipase, xylanase, protease and cellulase. In brief, point inoculation of bacterial species was done on respective agar medium and incubated for 24 h at 55 °C. After incubation, starch, carboxymethyl cellulose (CMC) and xylanase producing agar plate was flooded with Gram's iodine for observing zone of clearance around the bacterial colony. Zone of clearance for lipolytic and proteolytic activity was recorded on tributyrin agar and skim milk agar, respectively.

Table 1: Colony morphology and microscopic characters of thermobacilli

S. No.	Isolate code	Phenotypic characters		Bacterial species	Nucleotide accession no.
		Colony morphology	Cell morphology		
1	GBPI 3	Light yellow, smooth, slimy, circular, convex, 0.3-1.0 mm dia	0.6-0.8×3.2-4.5 µm, rod shaped, single, diplobacilli, short or long spiral chains	*Geobacillus stearothermophilus*	EU381182
2	GBPI 4	Light yellow, smooth, slimy, circular, convex, 7.5-9.0 mm dia	0.8-1.0×6.0-8.0 µm, rod shaped, single, diplobacilli, short or long spiral chains	*G. kustophilus*	EU381189
3	GBPI 13	Light yellow, smooth, slimy, circular, convex, 3-6 mm dia	0.6-0.8×4.5-5.0 µm, rod shaped, single, diplobacilli or short chains	*G. stearothermophilus*	EU381187
4	GBPI_16	Yellowish cream, smooth, slimy, circular, convex, 0.7-1.0 mm dia	0.8-1.0×6.0-8.0 µm, rod shaped, scattered or in clusters or short to long chains	*G. stearothermophilus*	FJ548759
5	GBPI_22	Creamish-white, smooth, convex and round colony, 2.0-10 mm dia	0.6-0.9×5.0-5.6 µm, rods in long chains	*Bacillus licheniformis*	KF862007
6	GBPI_25	Creamish-white, smooth, convex and round colony, 1.0-2.0 mm dia	0.8-1.0×7.0-7.5 µm, rods in long chains	*Paenibacillus ehimensis*	KF862010
7	GBPI_30	Off-white, smooth, convex-round colony, 1.0-2.0 mm dia	0.6-0.8×5.0-5.5 µm, rods in long chains	*P. ehimensis*	KF862013
8	GBPI_31	Creamish-white, irregular, convex-raised colony, 4.0-5.0 mm dia	0.5-08×4.0-5.5 µm, rods in long chains	*P. ehimensis*	KF862013
9	GBPI_35	Creamish-white, smooth, convex-round colony, 1.0-1.2 mm dia	0.6-0.8×4.5-5.0 µm, single rods	*P. ehimensis*	KF862018
10	GBPI _37	Creamish-white, smooth, convex-round colony, 1.0-3.0 mm dia	0.6-0.8×6.5-7.0 µm, single rods	*B. sonorensis*	KF862020

[13-15]

Quantitative production

The quantitative production of all five thermozymes was carried out under three different temperatures 45, 55 and 65 °C; at each temperature enzyme production was recorded at 5, 7 and 9 pH up to 72 h with an interval of 24 h.

Amylase assay

Amylase production was carried out in 250 ml conical flask containing 100 ml medium with following chemical composition in 1 liter: 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 and inoculated with 0.1 ml of 12 h old culture. The flasks were incubated at different temperatures (45, 55 and 65 °C) and pH (5, 7 and 9) for defined incubation time in static conditions. After incubation, biomass and enzyme activity was recorded at an interval of 24 h up to 72 h. All the experiments were conducted in triplicates.

Amylase activity was measured by using di-nitrosalicylic acid (DNSA) method [16]. The reaction mixture contained 0.3 ml crude enzyme, 0.5 ml different pH buffer, 0.2 ml of 1% (w/v) of corn starch as substrate and incubated at room temperature for 2 min. After incubation, 1 ml of DNSA was added to reaction mixture and kept in boiling water bath for 10 min. After boiling, reaction mixture was allowed to cool at room temperature and change in colour was measured at 540 nm against substrate blank as well as enzyme blank. Enzyme activity was recorded in terms of the amount of enzyme required to release 1.0µg of glucose per min under specified assay conditions.

Lipase assay

Lipase producing medium contained $NaNO_3$3.0g, K_2HPO_4 0.1g, $MgSO_4.7H_2O$ 0.5g, KCl 0.5g, $FeSO_4.7H_2O$ 0.01g, yeast extract5.0g and 1% olive oil in 1 liter and inoculated with 0.1 ml of 12 h old culture. Cell biomass and lipase activity was observed at an interval of 24 h up to 72 h. Lipase activity was determined following Pinsirodom and Parkin [17] method. In brief, reaction mixture was prepared by mixing 1 ml of 4-nitrophenyl laurate (*p*NPL) as lipase substrate, 1 ml buffer of desired pH and 0.4 ml crude enzyme extract, incubate reaction mixture at room temperature for 2 min. Thereafter, absorbance was recorded at 410 nm and amount of enzyme expressed in terms of enzyme unit which was defined as the µmol of *p*NP released per minute.

Xylanase assay

Xylanase production by thermophilic bacterial isolates was recorded by incubating bacterial cells in xylanase producing broth medium having composition in liter as follows: yeast extract 10g, glucose 10g, tween80 1 ml, brich wood xylan10g, 100 ml of medium was distributed in 250 ml conical flask. The medium was inoculated with 0.1 ml of 12 h old culture and incubated at different temperature and pH under static conditions for desired time period. After incubation period, broth medium was centrifuged at 10,000 rpm for 15 min and cell free supernatant was used as source of enzyme.

Xylanase activity was measured by using 1% xylan in desired pH buffer as substrate described by Bailey *et al.* [18] and quantity of reducing sugar released by the hydrolysis of xylan was observed by Miller [16]. In brief, reaction mixture contained 0.9 ml xylan solution and 0.1 ml crude extract; it was incubated for 2 min at room temperature. Following incubation, 1.5 ml of DNSA was added to reaction mixture and kept at 50 °C for 15 min. After boiling, reaction mixture was allowed to cool at room temperature and change in colour was measured at 540 nm. One unit of xylanase activity was defined as the amount of enzyme that required for the release of 1 µg of xylose per min.

Protease assay

Determination of protease production was done by incubating bacterial isolates in protease producing medium. The medium contained following composition per liter: yeast extract 5g, peptone 5g, glucose 5g, Na_2HPO_4 0.4g, Na_2CO_3 0.085g, $ZnSO_4$ 0.02g, $MgSO_4$ 0.02g and adjusted to desired pH. Broth was inoculated with 0.1 ml of 12 h old culture and incubated at different temperature and pH. After fermentation, broth medium was centrifuged at 10,000 rpm for 15 min and cell free supernatant was used for enzyme assay.

Protease assay was done by using 2 % casein in desired pH as substrate. Reaction mixture consisted 0.1 ml casein buffer and 0.1 ml crude enzyme, reaction was allowed to occur at 65 °C for 10 min in water bath. Afterward, reaction was stopped by adding 1 ml of 10% trichloroacetic acid (TCA). The mixture was then centrifuged at 10,000 rpm for 20 min after centrifugation; protein content was estimated by Lowry *et al.* [19]. One unit of protease activity was defined as the amount of enzyme that is required for the release of 1 µmole tyrosine per min.

Cellulase assay

Cellulase production was assayed using cellulase producing medium that contained carboxymethyl cellulose (CMC) 5 g, tryptone 2g, K_2HPO_4 4g, Na_2HPO_4 4g, $MgSO_4.7H_2O$ 0.2g, $CaCl_2.2H_2O$ 0.001g, $FeSO_4.7H_2O$ 0.004g. Each flask was inoculated with 12 h old culture and incubated at different temperature and pH. Following prescribed incubation period, cell free supernatant was prepared by centrifugation method and used as a source of crude enzyme.

Cellulase assay was conducted following Miller [16]. Briefly, reaction mixture contained 0.75 ml of buffer, 0.5 ml of crude and 0.25 ml substrate (1% CMC buffer) solution; it was allowed to react at 45 °C for 30 min and then incorporated with 1.5 ml of DNSA. All the tubes were then placed in boiling water bath for 10 min. Absorbance of colour change was recorded at 540 nm by using a UV/Vis spectrophotometer (Ultraspec 2100 pro, Amersham Biosciences). One unit of enzyme is defined as the amount of enzyme required for the release of 1 µg glucose per min.

Protein estimation

Total protein was estimated using Lowry's method [19].

Ammonium sulfate precipitation

The bacteria were grown in respective medium for obtaining desired amount of enzyme for its partial purification. After incubation, bacterial culture was centrifuged and cell free supernatant was subjected to ammonium sulfate precipitation at 4 °C (80 % saturation). The precipitate was dissolved in minimal amount of respective buffer of active pH and then dialyzed overnight against same buffer at 4 °C.

Enzyme activity and stability

Effect of temperature on the activity of thermophilic enzymes was observed by incubating reaction mixture at

different temperatures, ranged from 10-100 °C, in the preferred buffer system. Thermal stability of partially purified enzymes was observed by incubating crude enzyme at different temperatures for 1 h. Afterward, residual activity was recorded by analyzing the enzyme reaction at optimum temperature in buffer of respective pH as described earlier.

To determine the optimal pH for enzyme activity, enzyme assays were performed in different buffer system ranging from 3-10 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 mmol) used for different pH include citrate buffer (pH 3), citrate phosphate buffer (pH 4-5), sodium phosphate buffer (pH 6-7), Tris-Cl (pH 8-9) and glycine-NaOH buffer (pH 10).

Statistical analysis

Variation in enzyme activities was estimated by one way ANOVA followed by post hoc Tukeys HSD test to find significant differences between enzyme production at different physiological cultural conditions. Mean and standard error of three replicates was calculated using Microsoft Excel 2007 software.

RESULTS

Qualitative assays

Results on the production of extracellular hydrolytic enzymes, measured in terms of hydrolysis of substrate viz. starch, lipid, xylan, cellulose and casein, are presented in table 2. All the isolates were capable of hydrolyzing starch and lipids, nine isolates were able to hydrolyze xylan and cellulose and seven isolates hydrolyzed casein. On the basis of the results obtained in qualitative assays, the efficient bacterial species were selected for further quantitative assays as follows: GBPI_25 for amylase, GBPI 3 for lipase, GBPI_25 for xylanase, GBPI_35 for protease and GBPI 4 for cellulase.

Qualitative assays

On the basis of zone of clearance, GBPI_25 was selected for the quantitative estimation of amylase enzymes. The effect of different temperature and pH on amylase production with biomass was measured using starch broth in static conditions. GBPI_25 was found to be an efficient producer of amylase in the temperature range from 45 to 65 °C. Production of enzyme started after 24 h of incubation at all the temperatures while the maximum activity varied with respect to temperature and pH. Maximum activity (1217.86 U/ml) of amylase enzyme was observed after 24 h at 45 °C and 5 pH which showed significance difference ($p<0.05$) from other pH at same temperature (fig. 1). However, higher biomass production was achieved at 45 °C after 72 h of incubation at neutral pH (table 3).

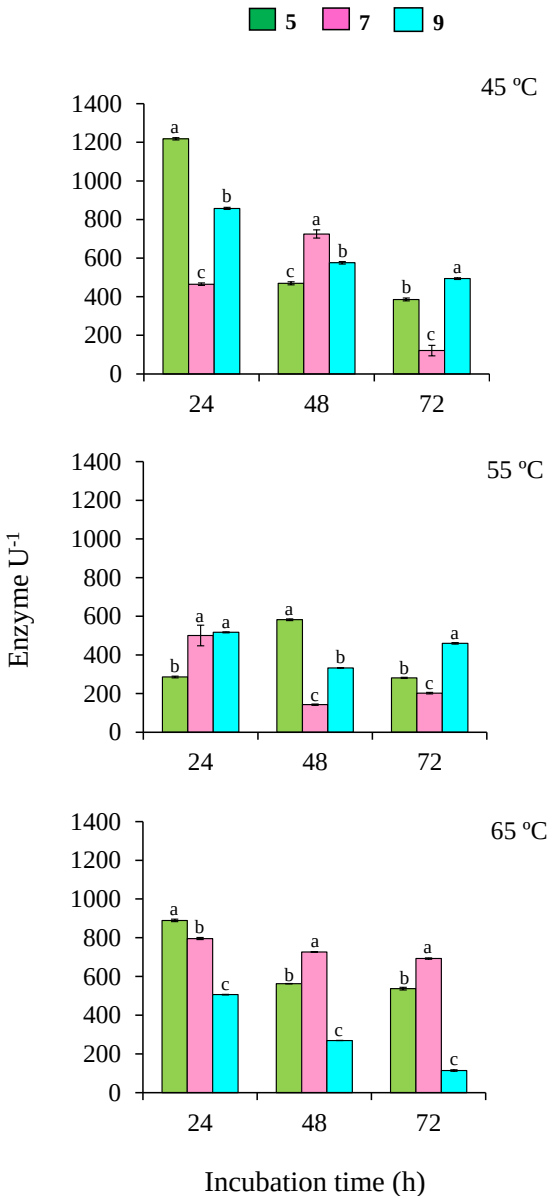

Fig. 1: Amylase production by GBPI_25 at different temperature and pH. Different alphabets in a bar group indicate significant difference ($p<0.05$). Error bar = standard error (n=3)

Table 2: Qualitative estimation of extracellular enzymes produced by thermobacilli

Zone of clearance (mm)						
S. No.	**Isolate Code**	**Amylase**	**Lipase**	**Xylanase**	**Cellulase**	**Protease**
1	GBPI 3	++	+++	+	++	-
2	GBPI 4	+	+	++	+++	+
3	GBPI 13	+	+	+	++	-
4	GBPI_16	++	+	+++	+++	+
5	GBPI_22	+	+	-	+	+
6	GBPI_25	+++	+	+++	+++	-
7	GBPI_30	+++	+	+	+	+
8	GBPI_31	+++	+	+	+	+
9	GBPI_35	+++	+	+	-	++
10	GBPI _37	++	+	+	+	+

(-= no zone,+=<2 mm,++= 3-5 mm,+++=>5 mm)

Table 3: Biomass production by thermobacilli at different temperature and pH

Enzyme	Isolate code	Temp. (°C)	pH	Bacterial biomass (O. D. at 600 nm)		
				24 h	48 h	72 h
			5	0.081±0.004^{a}	0.091±0.003^{a}	0.169±0.005^{b}
Amylase	GBPI_25	45	7	0.077±0.004^{a}	0.032±0.009^{b}	0.226±0.009^{a}
			9	0.018±0.001^{b}	0.091±0.004^{a}	0.186±0.004^{b}
			5	0.109±0.001^{a}	0.077±0.012^{b}	0.126±0.001^{b}
		55	7	0.093±0.008^{a}	0.126±0.009^{a}	0.176±0.003^{a}
			9	0.017±0.010^{b}	0.052±0.001^{b}	0.052±0.004^{c}
			5	0.056±0.001^{a}	0.048±0.003^{c}	0.146±0.009^{b}
		65	7	0.025±0.003^{c}	0.077±0.001^{b}	0.045±0.002^{c}
			9	0.042±0.001^{b}	0.124±0.002^{a}	0.220±0.005^{a}
			5	0.162±0.006^{b}	0.252±0.007^{a}	0.400±0.006^{b}
Lipase	GBPI3	45	7	0.217±0.012^{a}	0.155±0.003^{b}	0.197±0.003^{c}
			9	0.043±0.009^{c}	0.171±0.007^{b}	0.707±0.007^{a}
			5	0.012±0.002^{c}	0.283±0.005^{a}	0.282±0.007^{a}
		55	7	0.046±0.008^{b}	0.096±0.003^{b}	0.145±0.005^{c}
			9	0.533±0.005^{a}	0.107±0.005^{b}	0.732±0.010^{c}
			5	0.010±0.005^{c}	0.027±0.005^{b}	0.225±0.001^{a}
		65	7	0.127±0.006^{b}	0.039±0.004^{b}	0.109±0.004^{c}
			9	0.230±0.003^{a}	0.190±0.003^{a}	0.128±0.003^{b}
			5	0.088±0.005^{b}	0.111±0.001^{b}	1.203±0.004^{a}
Xylanase	GBPI_25	45	7	0.118±0.007^{a}	0.190±0.005^{a}	0.093±0.001^{c}
			9	0.056±0.002^{c}	0.229±0.006^{a}	0.314±0.001^{b}
			5	0.061±0.001^{c}	0.107±0.001^{a}	0.181±0.001^{b}
		55	7	0.190±0.005^{a}	0.085±0.007^{b}	0.138±0.004^{c}
			9	0.087±0.001^{b}	0.054±0.001^{c}	0.209±0.005^{a}
			5	0.055±0.003^{b}	0.099±0.001^{b}	0.132±0.001^{b}
		65	7	0.093±0.001^{a}	0.111±0.001^{ab}	0.150±0.003^{a}
			9	0.021±0.004^{c}	0.115±0.005^{a}	0.149±0.004^{a}
			5	0.296±0.006^{a}	0.204±0.001^{a}	0.118±0.003^{a}
Protease	GBPI_35	45	7	0.139±0.003^{b}	0.093±0.002^{b}	0.016±0.002^{c}
			9	0.135±0.001^{b}	0.089±0.005^{b}	0.166±0.004^{b}
			5	0.167±0.007^{b}	0.213±0.007^{a}	0.224±0.001^{b}
		55	7	0.312±0.001^{a}	0.116±0.001^{b}	0.015±0.001^{c}
			9	0.135±0.006^{c}	0.224±0.006^{a}	0.260±0.001^{a}
			5	0.034±0.005^{b}	0.032±0.001^{b}	0.067±0.001^{b}
		65	7	0.012±0.001^{c}	0.045±0.006^{b}	0.026±0.001^{c}
			9	0.233±0.003^{a}	0.247±0.003^{a}	0.720±0.006^{a}
			5	0.015±0.001^{b}	0.009±0.003^{b}	0.138±0.001^{c}
Cellulase	GBPI 4	45	7	0.055±0.001^{a}	0.096±0.006^{a}	0.375±0.005^{a}
			9	0.018±0.001^{b}	0.020±0.001^{b}	0.316±0.007^{b}
			5	0.013±0.001^{c}	0.069±0.004^{b}	0.046±0.002^{b}
		55	7	0.085±0.001^{a}	0.093±0.001^{a}	0.054±0.003^{b}
			9	0.024±0.001^{b}	0.066±0.001^{b}	0.104±0.001^{a}
			5	0.011±0.000^{b}	0.067±0.004^{a}	0.044±0.002^{c}
		65	7	0.031±0.001^{a}	0.045±0.001^{b}	0.201±0.003^{a}
			9	0.012±0.001^{b}	0.034±0.001^{c}	0.173±0.001^{b}

Values are mean±SE of triplicates. Mean with different alphabets are significantly different on same incubation time

GBPI3 showed lipase production at all the temperature and pH conditions while higher concentration of enzyme was recorded at pH 9 at all the temperatures. At 65 °C, maximum amount of enzyme (22.59 U/ml) was achieved after 48 h of incubation, which was significantly different ($p<0.05$) from other pH (fig. 2). Maximum cell biomass was recorded at the optimum growth temperature (55 °C) and pH (9), after 72 h of incubation.

Considering the good observation on xylanase production in qualitative assays, GBPI_25 was subjected for its quantitative estimation at temperature ranged from 45 to 65 °C and pH 5 to 9. Enzyme production was observed after 24 h of incubation, although the higher activity varied with different temperature and pH. The significantly higher (<0.05) amount (98.07 U/ml) of xylanase was found at 9 pH and 45 °C after 48 h of incubation. Biomass was produced maximum after 72 h of incubation at 45 °C and pH 9. The time course of xylanase production by GBPI_25 is shown in Fig.3.

In case of protease enzyme, maximum zone of clearance was recorded in the thermophilic isolate GBPI_35. Minimum enzyme activity 0.42 U/ml was revealed at the optimum growth temperature (55 °C) and in acidic pH (5). Maximum enzyme activity (16.66 U/ml) was observed at 55 °C after 24 h incubation under alkaline pH (9) which was significantly different ($p<0.05$) from pH 5 and 7 (fig. 4). The maximum biomass was recorded at 65 °C and pH 9 after 72 h of incubation (table 3).

Out of three temperatures, GBPI 4 showed increased cellulase production at suboptimal growth temperature (45 °C)

followed by production at 55 °C under acidic (pH 5) environment. In case of 65 °C, GBPI 4 showed decrease in production of cellulase enzyme at all the pH conditions. The maximum enzyme production i.e. 108.68 U/ml was achieved at 45 °C and pH 5 conditions after 72 h of incubation which was significantly different ($p<0.05$) from the other pH under consideration, but at the same temperature in static conditions. The minimum production (2.34 U/ml) of cellulase was recorded at 65°C (fig. 5). The higher concentration of cell biomass was produced at 45 °C and pH 9 after 72 h (table 3).

Enzyme activity and stability

The amylase extracted from GBPI_25 showed a wide temperature range (10-90 °C) with maximum activity at 50 °C that got decreased subsequently. However, the enzyme exhibited maximum (87.52 %) stability at 40 °C after 1 h of incubation (fig. 6A). Optimum pH for amylase activity was recorded between 5 to 7, and being maximum at 5 (Fig.6B).

In case of lipase, GBPI 3 possessed wide range of temperature (10-80 °C) as presented in fig. 6C. Both the enzyme activity as well as the stability was found maximum at 40 °C. Optimum pH for activity as well as the stability of lipase was recorded at 5 (fig. 6D).

The xylanase extracted from the thermophilic isolate GBPI_25 exhibited temperature range between 10 to 100 °C for its activity as well as stability. The enzyme was more active at 50 °C followed by 40 °C (96.60 %) while maximum stability was obtained at 40 °C. Above and below 40°C, the stability of xylanase was found to be decreasing continuously. Similar to amylase enzyme from the same bacterial isolate, it also exhibited maximum activity and stability at pH 5. Effect of temperature and pH on the activity as well as stability of xylanase are presented in fig. 6EandF, respectively.

The optimum temperature of protease enzyme activity isolated from GBPI_35 was found to be maximum at 60 °C, and as the temperature raised or declined there was decrease in enzyme activity. However, maximum enzyme stability was recorded at 40 °C (fig. 6G). Highest protease activity and stability was obtained at 5 pH (fig. 6H).

Maximum activity of cellulose produced by GBPI 4 was revealed at 50 °C. Further increase in temperature resulted in decreased activity of the enzyme. On the other hand, stability of cellulase was recorded at 40 °C. Cellulase also showed similar stability at 30 and 60 °C (fig. 6I). This enzyme showed the highest activity as well as stability at neutral (7) pH (fig. 6J).

Microorganisms from extreme climatic conditions are being recognized as potential source of commercially important enzymes. Thermozymes, in particular, bear numerous advantages over mesophilic enzymes such as the activity and effectiveness under extreme temperature, pH, pressure and high substrate concentration [20]. The present study deals with the role of temperature and pH on the production of different thermozymes for their biotechnological applications as well as ecological resilience aspects.

The production of amylase enzyme from GBPI_25 showed higher activity at suboptimal growth temperature (45 °C) and acidic pH after 24 h of incubation. Similar incubation temperature and time was reported in earlier studies in *Bacillus* sp. and in *B. licheniformis* [21, 22], respectively.

Production of the higher concentration of amylase under the influence of temperature in acidic as well as alkaline conditions has been reported [23, 24]. The pH optima, in the present study, for enzyme activity and stability was similar as described by Asoodeh *et al.* [25]. *Geobacillus stearothermophilus* HP3 has been reported for production of novel thermostable and alkali tolerant amylase [26]. Amylase production by *Geobacillus* sp. GJA1 isolated from a hot spring located in Tapovan area of Uttarakhand (IHR) has also been studied by Jugran *et al.* [27]. In comparison to neutral α-amylases; acid stable amylases have 30 % less acidic and basic amino acids which play key role in protection of electrostatic repulsion of charged groups at acidic condition, and help in the stability of the protein and reduces risk of contamination. The major drawback of most of the known acid stable amylases is that they lack thermostability at high temperature, while present study showed maximum stability at 50 °C which is likely to be helpful in reducing this drawback [5].

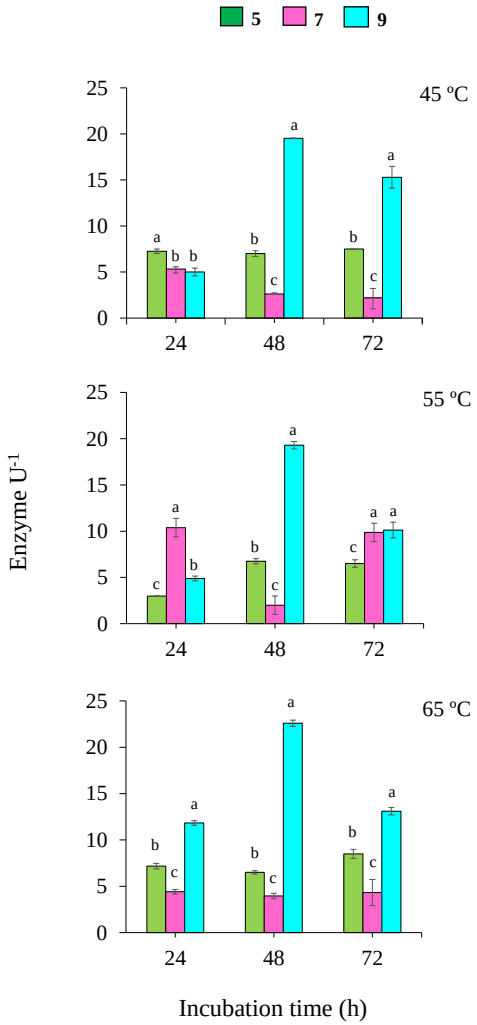

Fig. 2: Lipase production by GBPI 3 at different temperature and pH. Different alphabets in a bar group indicate significant difference ($p<0.05$). Error bar = standard error (n=3)

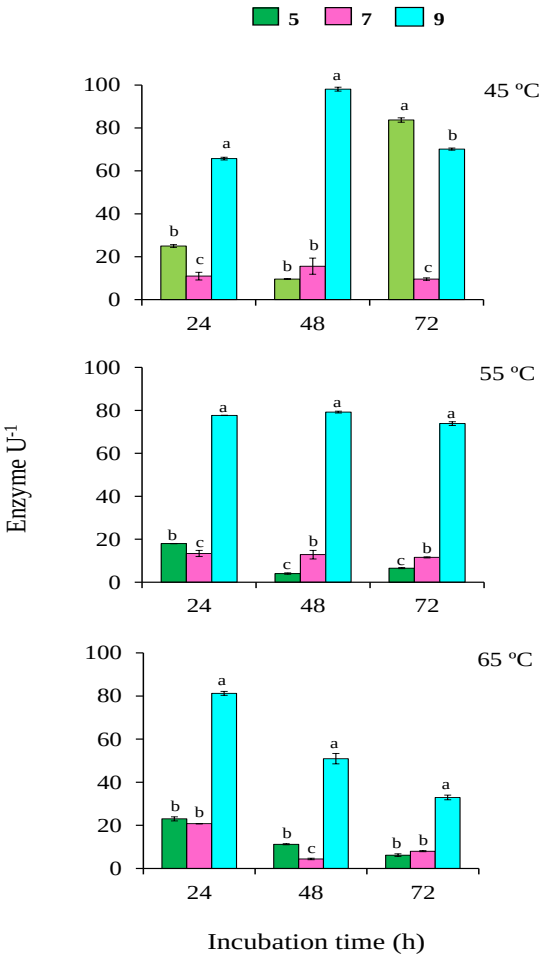

Fig. 3: Xylanase production by GBPI_25 at different temperature and pH. Different alphabets in a bar group indicate significant difference (p<0.05). Error bar = standard error (n=3)

Lipase production from GBPI 3 was observed higher at their suboptimal (65 °C) temperature and alkaline pH (9). The identical temperature for lipase production was reported from *G. stearothermophilus* [10], while 60 °C was optimum temperature for lipase production reported from *G. thermleovorance* [28] and *Aneurinibacillus thermoaerophilus* [29]. Berekaa *et al.* [30] reported that the production of lipase enzyme from thermophilic bacteria occurred maximally between temperatures 50 to 70 °C. Similar pH was also documented in thermophilic *Anoxybacillus flavithermus* [31] as well as in psychrophilic strain of *Pseudomonas proteolytica* [32]. Crude lipase was found active along a wide temperature and pH range and similar range of pH was also reported in a thermo-alkaline *Staphylococcus aureus* by Bacha *et al.* [33]. Maximum stability and activity in lipase production was also recorded at suboptimal culture (40 °C temperature and 5 pH) conditions which is similar to lipase from *Pseudomonas gessardii* [34]. According to Ramani *et al.* [34], at this pH and temperature, lipase was suitable for adsorption on the oil–water interface and helpful in the opening of block lid from the active site of the enzyme to lower the activation energy of hydrolysis.

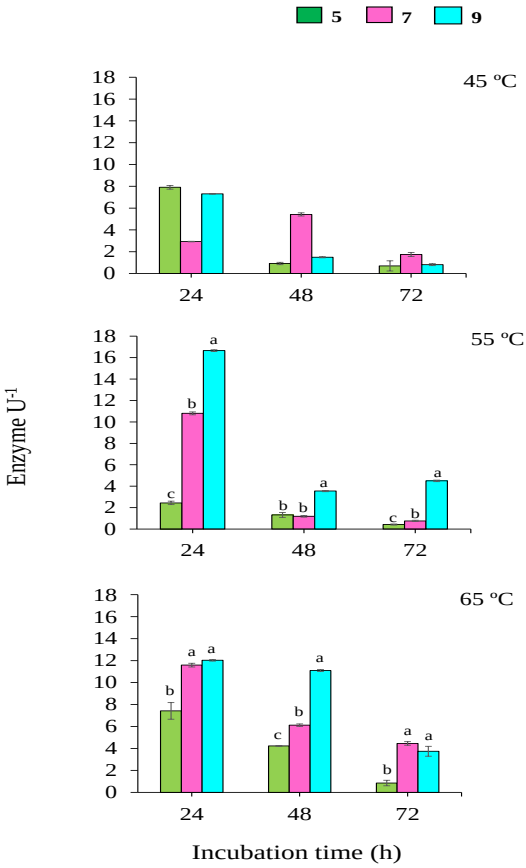

Fig. 4: Protease production by GBPI_35 at different temperature and pH. Different alphabets in a bar group indicate significant difference (p<0.05). Error bar = standard error (n=3)

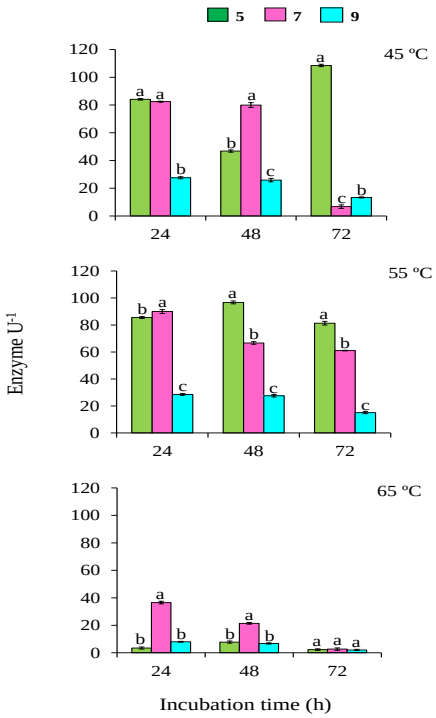

Fig. 5: Cellulase production by GBPI 4 at different temperature and pH. Different alphabets in a bar group indicate significant difference (p<0.05). Error bar = standard error (n=3)

DISCUSSION

The present study showed higher production of xylanase at alkaline pH. However, different optimum temperature has been observed in various thermophilic isolates namely *G. stearothermophilus* and *B. halodurans* [35, 36]. Xylanase from bacterial sources are active and stable in a wide range of pH and temperature conditions. Xylanase enzyme exhibited its optimal activity at wide range of incubation conditions, such as temperature from 30 to 60 °C and pH from 5.0 to 9.0 [37]. The present investigation revealed difference between the optimum culture conditions for enzyme production and its activity. Similar observations were also observed in *B. mojavansis* [38].

In case of protease enzyme secreted by thermophilic isolates GBPI_35, the temperature profile showed higher production at their optimum growth temperature i.e. 55 °C and alkaline pH conditions. A number of studies have indicated that the optimum production of protease enzyme occur at identical alkaline pH under different temperature conditions [39, 40]. According to Hadder *et al.* [41], protease enzyme having high temperature and alkaline pH activity make it ideal for detergent industries. However, enzyme activity and stability, observed in present study, is entirely different from the previous studies [38, 42].

In case of cellulase, GBPI4 produced higher concentration at acidic pH and 45 °C. The same pH was also recorded in *Gluconacetobacter* sp. Gel_SEA623-2 by Kim *et al.* [43]. However, in most of the cases maximum production of cellulase was recorded in alkaline pH [44, 45]. Partially purified enzyme showed wide temperature and pH range for its activity and stability with optimum 50 °C and acidic pH 7. In high temperature as well as mild alkaline conditions, production of cellulase may be useful in industrial applications [46].

CONCLUSION

In the present investigation, different thermophilic isolates have been screened and demonstrated for their ability to produce enzymes at varying pH and temperature conditions. Preliminary screening of such microorganisms that are isolated from extreme environments is essential in view of harnessing their economic importance. Promising strains can be further engineered using advance technologies for their commercial utilization. All the thermophilic bacteria under study showed unusual properties to tolerate wide range of pH and temperature which may be beneficial in a number of biotechnology based industries.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest

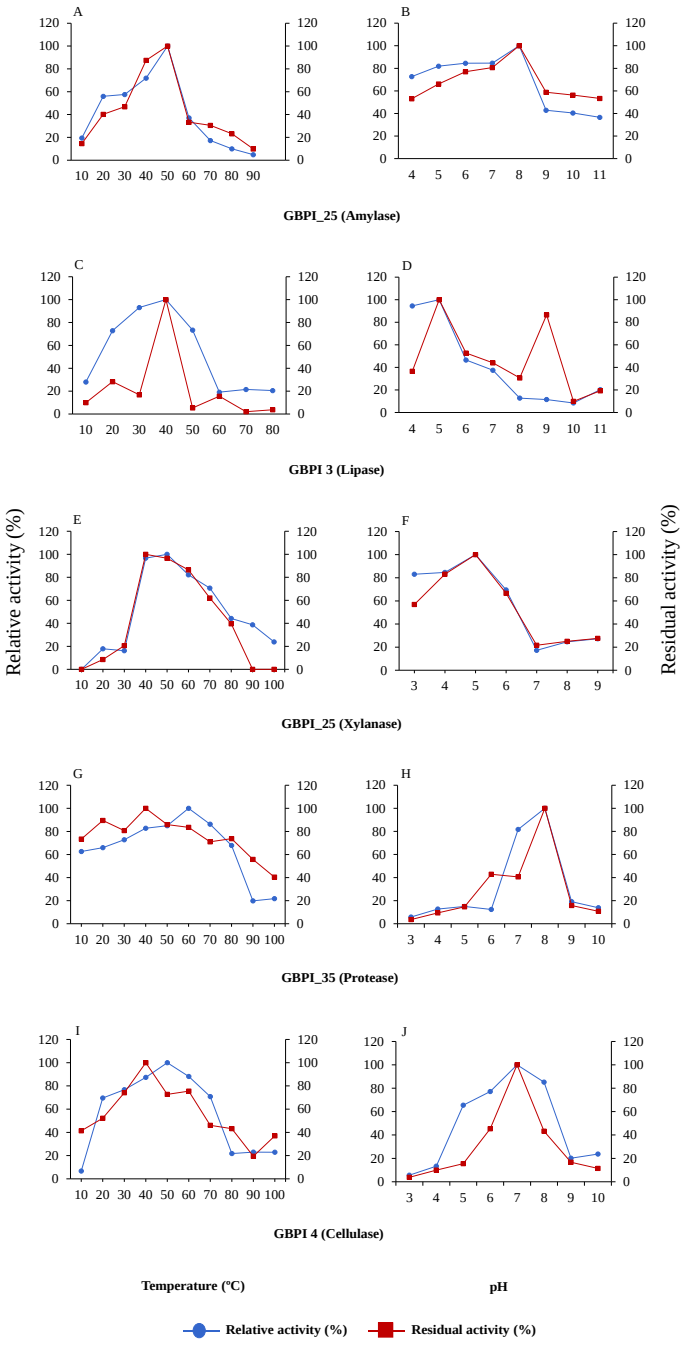

Fig. 6: Effect of temperature and pH on activity and stability of thermozymes produced by thermophilic bacterial species (AandB) GBPI_25 (amylase), (CandD) GBPI 3 (Lipase), (EandF) GBPI_25 (Xylanase), (GandH) GBPI_25 (Protease), (IandJ) GBPI_25 (Cellulase)

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