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Extraction, purification and characterization of amylase, protease, lipase of halophilic bacterial isolates from Bordi, India

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Abstract

Extremely halophilic isolates were obtained from mangrove vegetation of west coast Bordi region, Maharashtra India. All six isolates were selected for further investigation on the basis of their potential of producing enzymes amylase, protease and lipases respectively. Production of enzyme from isolates was performed using basal medium with starch, skimmed milk and tributyrin. Ammonium sulphate salt precipitation and dialysis was performed to obtain partially purified crude samples of enzymes. Activity of crude enzymes was determined in unit/ml by using standard methods. Also the effect of pH and temperature on enzyme activity was also determined.

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Introduction

Halophilic prokaryotes has been found in a wide range of saline environment within various geographical locations including salt lakes, marine salterns and saline soils (de la Haba, R.R et al.,2011, Lizama, C et al.,2001). Hypersaline environments host a considerable diversity of extremely halophilicarchea as well as halophilic and halotolerant bacteria (Oren, A., 2002). In the past few years, the microbial diversity of such hypersaline environment has been extensively explored using both culture dependent and culture independent techniques. (Benlloch, S et al.,1995, Borsodi, A.K et al.,2013, Burns, D.G et al.,2004, Youssef, N.H et al.,2012, Boutaiba, S., Hacène et al.,2011, Oren, A., 1983, Oren, A., 2014).

Halophiles provide a potential source of novel enzyme that function under salt stress condition, such as lipases, proteases, amylases, gelatinase and xylanases with polyextremophilic properties. (DasSarma S, DasSarma P., 2015). Halophilic enzymes are capable of functioning under high salt concentration, wide range of temperature and pH at which other protein denature. Certain enzymes that halophiles synthesize are useful for bioremediation of pollutants in saline habitats (Dastgheib SM et al.,2011). The halophilic enzymes have also been used in many industries including pharmaceutical, textile, detergent, baking, paper and pulp industries (Sarwar MK, Azam I, Iqbal T 2015).

A number of halotolerant and halophilic bacteria and archea such as *Bacillus*, *Halobacillus*, *Halomonas*, *Salinobacillus*, *Haloarcula* and *Haloferax* have been explored for

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their ability to hydrolyze enzymes like proteases, amylases and lipases (Bozic N, Ruiz J, Lopez-Santin J et al 2011). Halophilic enzymes are also considered as important biocatalyst under low water conditions such as hypersaline environment and non-aqueous media (Marhuenda-Ege FC, Bonete MJ 2002).

The current investigation focuses on (i) Isolating moderate halophiles from saline habitat, such as coastal regions of Bordi (Maharashtra) (ii) screening for industrially important enzymes (particularly amylases, lipases, and proteases), and (iii) studying few properties of these enzymes.

Materials and Methods

The present study was carried out on potential of screening, extraction, purification and characterization of industrially important enzymes from previously isolated and identified halophilic bacteria namely *Virgibacillusdokdonesis* DSW-10(T), *Kocuriaflava* HO-9041(T), *Halomonaspiezotolerans* NBT06E8(T),*Halolactibacillusmiurensis* DSM 17074(T), *Oceanobacilluscaeni* S-11(T), *Planococcusplakortidis* DSM 23997(T). These isolates were denoted in this study as isolate BB, BC_4, BJ, BK, $BS_2$13, BS_6 respectively.

Screening of extracellular hydrolytic enzymes

The extremozymes production from extremophilic microbes can be completed using the standard method and media. Microbes for enzyme production under normal as well as extreme conditions can be screened using diffusion agar plate of basal medium composition: Yeast extract 1 gm, KH_2PO_4 1 gm, $MgSO_4.7H_2O$ 0.1 gm, $CaCl_2.H_2O$ 0.05 gm, NaCl 10 gm, $NaCO_3$ 1 gm, 18 gm/ lit agar supplemented with different substrates such as starch (0.25%), Skimmed milk(10% w/v) and tributyrin (1%) (Divjot Kour et al.,2019).

Amylase

On basal medium supplemented with 0.25 % starch, isolates were spot inoculated and kept for incubation for 72 hrs. After incubation starch hydrolysis was detected by flooding plates with iodine-potassium iodide solution also known as Lugol's iodine solution containing 1 gm of iodine crystals, 5 gm potassium iodide and 330 ml distilled water. Clear zone around the colony indicates hydrolysis of starch (Montalvo-Rodriguez, R et al.,1998).

Protease

Proteolytic or caseinolytic activity of the microbes was tested in skimmed milk plates of basal medium with skimmed milk 10% w/v. Clear zone around the growth were considered as an indication of protease activity. For some microbes required more time period for hydrolyzing activity (Gonzalez, C et al.,1978).

Lipase

Lipase activity detected using basal medium containing 1% tributyrin (v/v). clear orange colored fluorescent halos around colonies were considered as indication of lipase activity. Here Rhodamine B (0.005%) was used and plates observed under UV light in chamber (Kouker, G. and Jaeger, K.-E. 1987, Boutaiba, S et al.,2006).

Production of enzymes

Basal medium with starch(0.25 %), skimmed milk(10 % w/v) and tributyrin(1 %) was used for the production of enzymes from six isolates. All the isolates were inoculated in the

Erlenmeyer flask containing 50 ml basal medium with substrates. Flasks were incubated on rotary shaker at 37^{0}C temperature and speed was maintaining 120 rpm for 8 days.

Purification of enzymes

The cultured broth centrifuged for 10min at 13000 Xg in 4^{0}C. Enzymes were precipitated by 80% saturated ammonium sulphate. The mixtures were left overnight at 4^{0}C. The precipitate was centrifuged for 30 min at 12000Xg and dissolved in 100 mMTris-HCl buffer (pH 8) and dialyzed to obtain crude partially purified enzymes. These crude and partially purified enzymes were used for determination of enzyme assay and activity at variable pH and temperature (Chakraborty S et al.,2009, Hmidet N et al.,2008).

Enzyme assay

Amylase assay

In this assay soluble starch 1% was used as substrate. If amylase digests starch, the maltose was released. Di-nitro salicylic acid (DNSA) react with maltose to generate deep orange red colored complex on boiling for 10-15 min, which was measured as an absorbance value on UV-Visible spectrophotometer (Systronics 117) at 540 nm. A maltose standard calibration curve was performed and constructed to determine amount of maltose released after the amylolytic activity. A series of maltose standard solution at different concentrations (100-1000µM) were prepared from the Maltose stock solution with deionized water. 1ml of properly diluted (in acetic acid buffer pH 4.9) crude enzyme was incubated for 15min at 40^{0}C with 1 ml of soluble starch solution 1% w/v. The produced quantity of reducing sugar released from starch was determined by taking absorbance at 540 nm. As unit of activity (U) of the enzyme amylase is arbitrarily appointed, the quantity of enzyme required for the production of 1µM of maltose in 1 min, when the enzyme was incubated along with the substrate at pH 4.9 and temperature 40^{0}C (Plummer, D 1978, Harvey,R 2005, Miller GL 1959).

Protease assay

In this assay, β-casein was used as substrate. If protease digest casein, the aminoacid tyrosine is liberated along with free other peptide fragments. Folin's reagent react with free tyrosine to generate a blue colored product, which was measured as an absorbance value on UV-Visible spectrophotometer (systronics 117) at 660 nm. A tyrosine standard calibration curve was performed and constructed to determine amount of tyrosine released after the proteolytic activity. A series of tyrosine standard solution at different concentrations (40-200 µg/ml) were prepared from the L-tyrosine stock solution with deionized water. 1 ml substrate casein (1%) incubated with 1 ml crude enzyme at 400C for 30min. Addition of 5% TCA 5ml in reaction mixture allowed it to stand for 10 min at room temperature, then addition of Folin's reagent 0.5ml. Measure the absorbance at 660 nm against blank without enzyme. The unit of activity of protease calculated as the amount of enzyme that release one micromole of the product per milliliter of the sample per minute under standard reaction conditions (Plummer, D 1978, Harvey,R 2005, Peterson 1977).

Lipases assay

To test for lipase activity, the crude enzyme preparation was assayed titrimetrically. To 3ml of phosphate buffer (pH 7.5) was added 1ml of substrate (olive oil), 2.5 % gum Arabic and 0.5 ml of crude enzyme. The reaction was performed under continuous agitation for 5min. The reaction was terminated by addition of 25 ml acetone:ethanol (1:1) and librated fatty acid was titrated against 50mM NaOH. Indicator used for titration was thymopthalein

0.9%. Mixture was titrated until light blue colour appears. One unit of lipase activity was equivalent to the amount of enzyme that catalyzes the release of 1µM of fatty acid from the substrate per milliliter in 1 min under the standard assay conditions (Gupta, N., Mehra, G. and Gupta, R., 2004).

Effect of pH and Temperature on enzyme activity

The crude enzyme was used for checking activity at variable pH and temperature. pH range used for all three enzymes was 6 to 10 with difference of 0.5. the enzyme stability is depending on the optimum pH. Each enzyme has a region of optimum pH for stability. The optimum pH was determined by incubating the enzyme with substrate at pH 6 to 10. The enzyme activity was calculated at each pH on which crude enzyme incubated. Curve was plotted of enzyme rate of reaction against the different pH at which the enzyme catalytic reactions are incubated. From the curve optimum pH which had given the maximum activity of the enzyme was noted.

Enzyme assay with suitable substrate for all three enzymes was performed at a range of temperature 4^0C, 25^0C, 37^0C and 55^0C according to method describe above. The maximum enzyme activity was found by plotting graph temperature versus enzyme activity.

Results and Discussion

All the isolates was used for study related to their extracellular enzyme producing ability were isolated from mangrove rhizosphere and have ability to show growth at high salt concentration up to 15% NaCl. These isolates were isolated from Bordi region west coast Maharashtra India.

Screening of enzyme was performed with the help of modified basal medium containing 0.25% starch, 10% (w/v) skimmed milk and 1% (v/v) tributyrin. Already identified isolates were shown potential of producing all the three enzymes. Observation of starch hydrolysis was performed by using Lugol's iodine solution, casein hydrolysis was observed against the brightly illuminated light and lipid hydrolysis by using 0.005% Rhodamine B under UV chamber (Table 1).

Enzymes were produced in large quantity by using same media which was used for screening. After incubation of 7 days fermented broth was centrifuged and used for ammonium sulphate precipitation. 80% (w/v) salt was used for obtaining the maximum precipitate. Precipitate was dissolved in 100mM Tris-HCl buffer (pH 8.0). Dissolved precipitate used for dialysis and partially purified enzyme used for assay and for checking activity.

Activity of enzyme amylase was determined by using standard plot of maltose (1000mM). Maximum amylase activity on 1% starch was shown by isolate BJ (121.68U/ml) and lowest activity by isolate BB (43.74U/ml) respectively. Protease activity was determined by using standard plot of L-Tyrosine (200mcg/ml). Maximum activity noted for isolate $BS_2$13 (43.74 U/ml) and lowest activity for isolate BJ (8.33U/ml). Lipases activity was determined by using olive oil and Arabic gum mixture. Maximum activity noted for isolate BS_6 (187 U/ml) and lowest activity given by isolate BB (86 U/ml) (Table 2) (Figure 1).

Activities of enzymes were also determined on different pH condition. After calculating the activity of each enzyme on different pH condition it was found that pH 8.5 is suitable for production of enzymes as most of them showed maximum activity at pH 8.5. Isolate BB and $BS_2$13 showed maximum protease activity at pH 8.5 while $BS_2$13, BJ and BS_6 showed maximum lipases activity at pH 8.0. (Figure 2,3,4) Activity of enzymes was also

determined at variable temperature; it was found that all enzymes have been given maximum activity at 37^0C and though even at 50^0C enzyme is not losing a lot activity (Figure5,6,7).

Table 1. Screening of enzyme production by Isolates

Isolate	Name of isolate	Amylase	Protease	Lipase
BB	*Virgibacillusdokdonesis* DSW-10(T)	+	+	+
BC_4	*Kocuriaflava* HO-9041(T)	+	+	+
BJ	*Halomonaspiezotolerans* NBT06E8(T)	+	+	+
BK	*Halolactibacillusmiurensis* DSM 17074(T)	+	+	+
$BS_2$13	*Planococcusplakortidis* DSM 23997(T)	+	+	+
BS_6	*Oceanobacilluscaeni* S-11(T)	+	+	+

Table 2. Enzyme activity of Isolates

Isolate	Name of isolate	Amylase U/ml	Protease U/ml	Lipase U/ml
BB	*Virgibacillusdokdonesis* DSW-10(T)	43.74	32.15	86
BC_4	*Kocuriaflava* HO-9041(T)	63.02	11.82	129
BJ	*Halomonaspiezotolerans* NBT06E8(T)	121.68	8.33	165
BK	*Halolactibacillusmiurensis* DSM 17074(T)	101.12	12.91	104
$BS_2$13	*Planococcusplakortidis* DSM 23997(T)	84.38	43.74	143
BS_6	*Oceanobacilluscaeni* S-11(T)	76.39	10.06	187

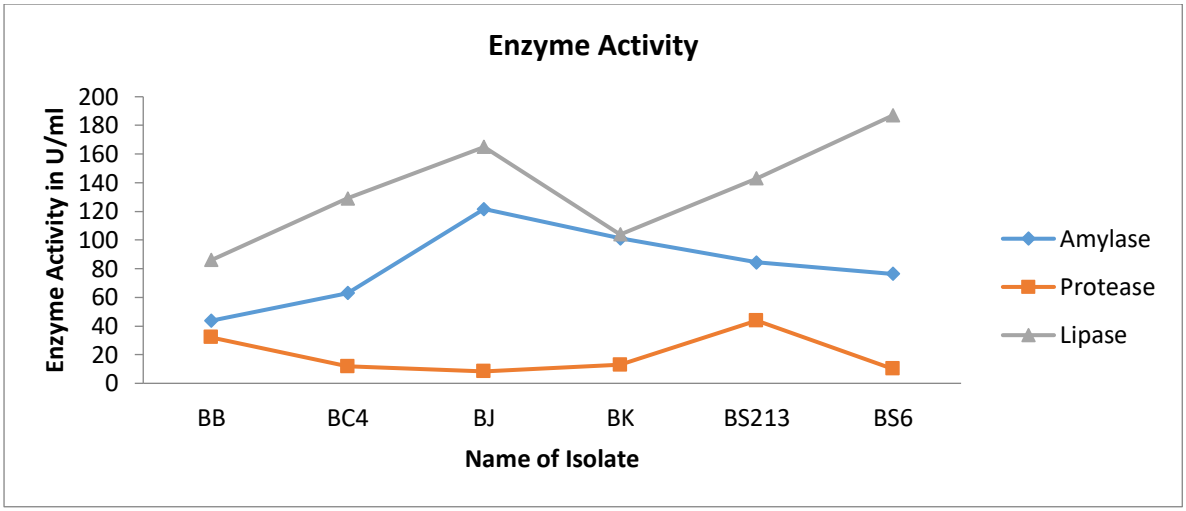

Figure 1. Enzyme activity

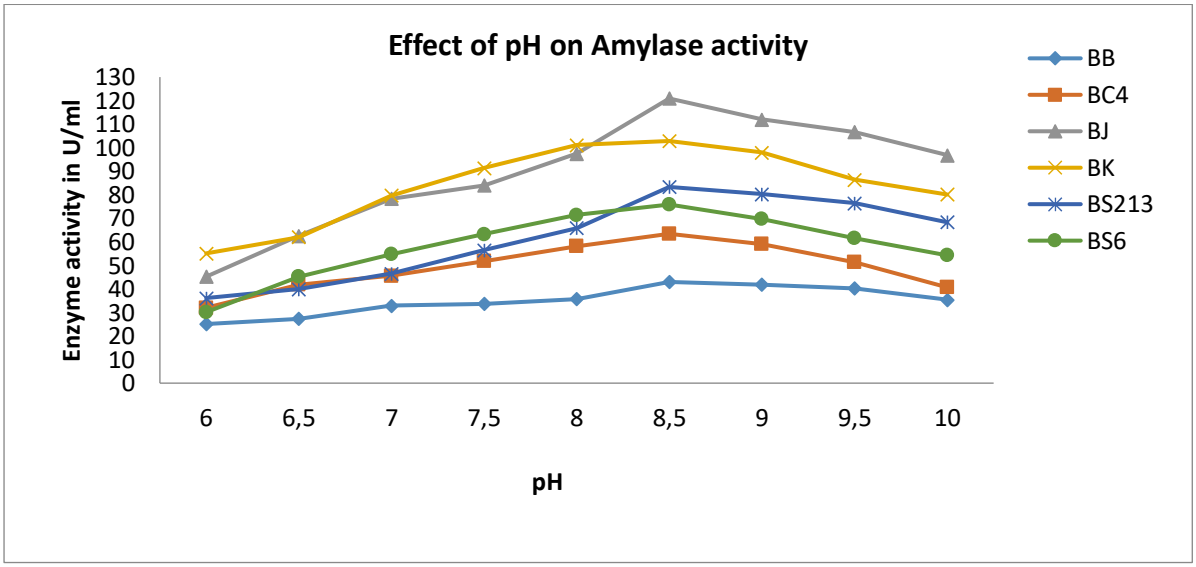

Figure 2. Effect of pH on amylase activity

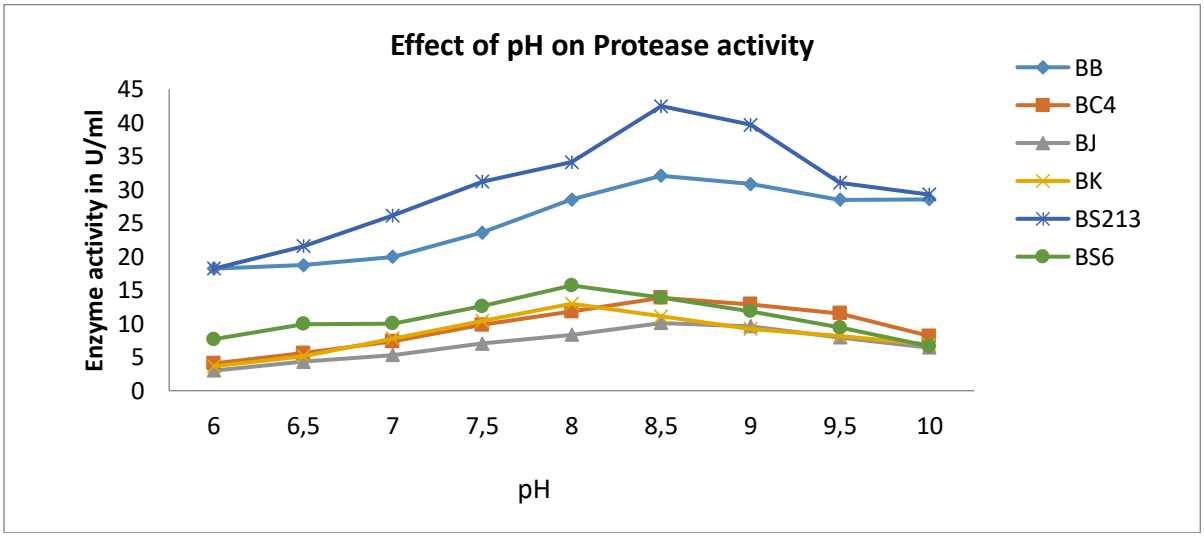

Figure 3. Effect of pH on Protease Activity

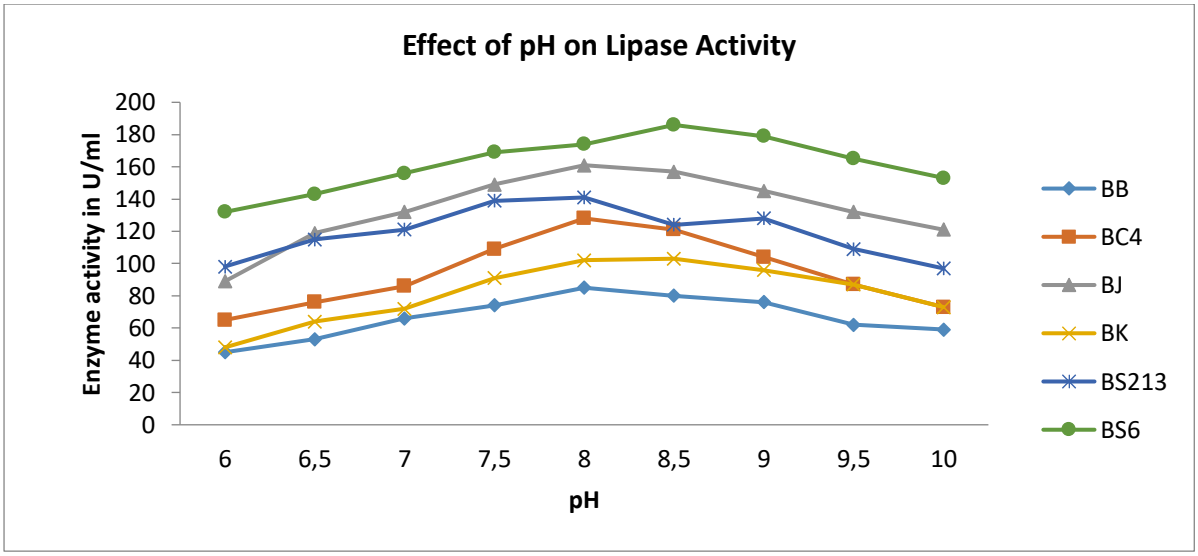

Figure 4. Effect of pH on Lipase Activity

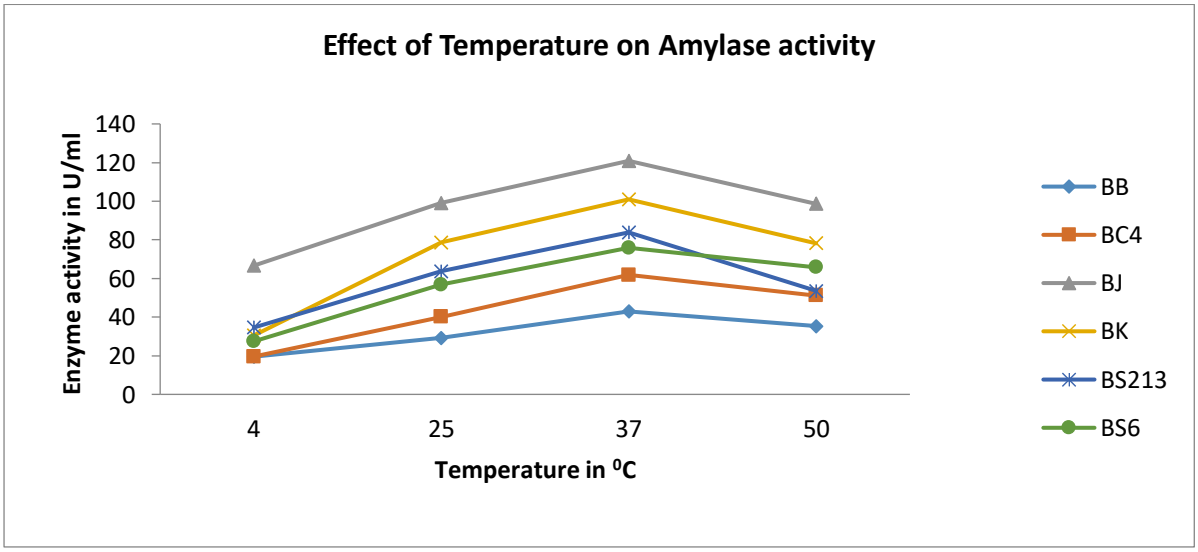

Figure 5. Effect of temperature on Amylase activity

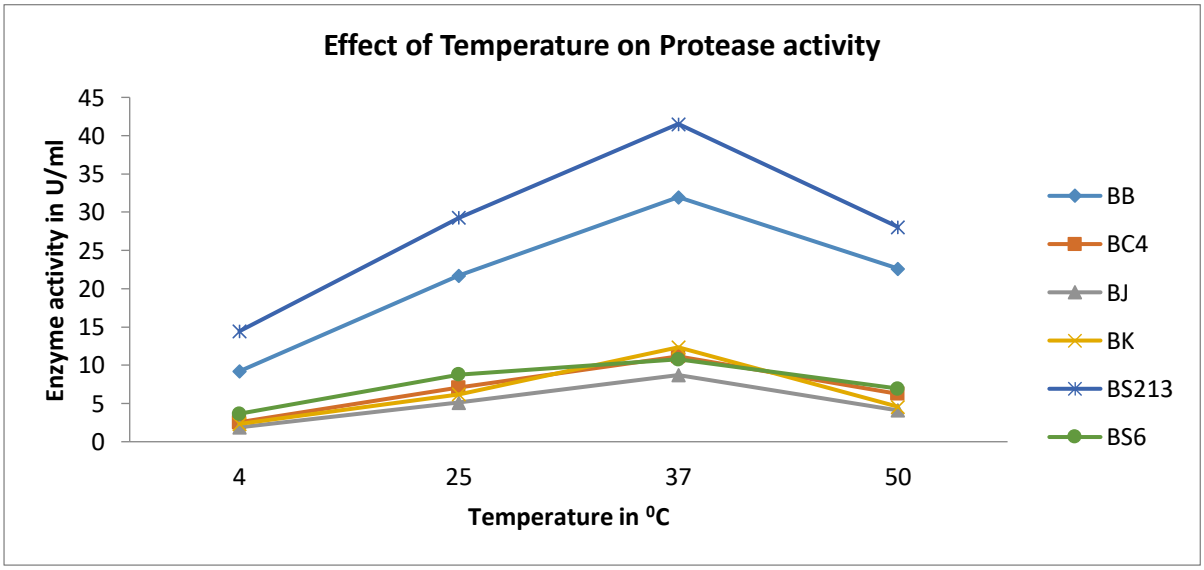

Figure 6. Effect of Temperature on Protease Activity

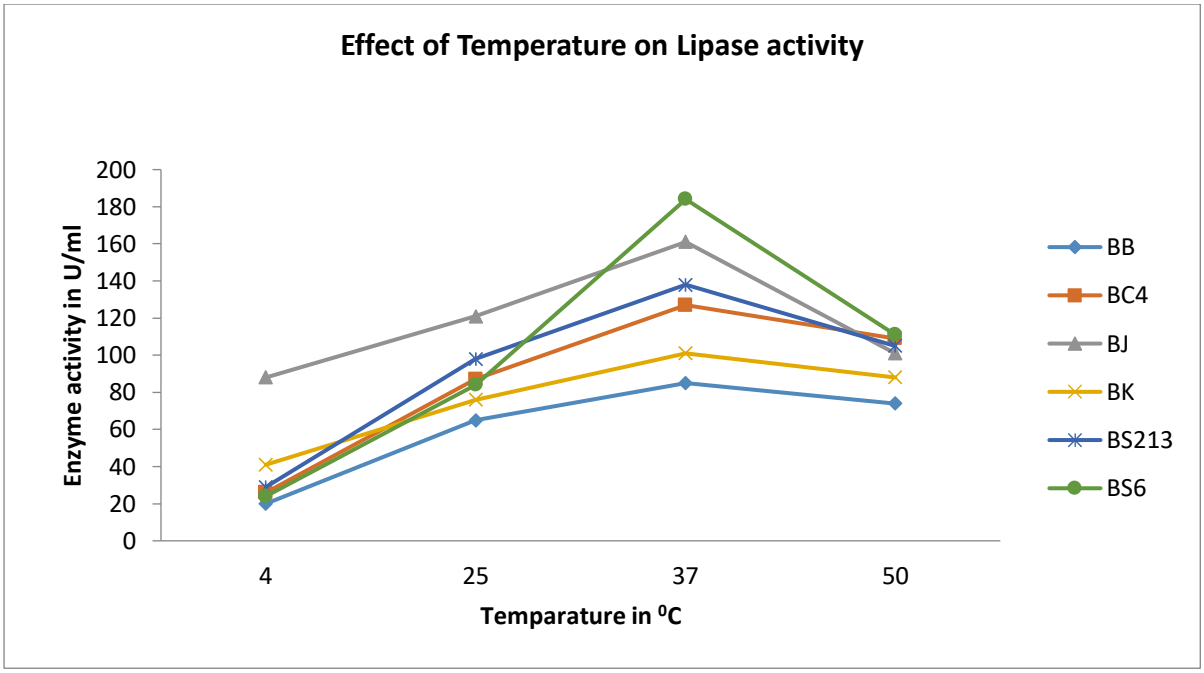

Figure 7. Effect of Temperature on Lipase Activity

Conclusions

In recent years, the enzymes from extremophiles have been in very high demand in various sectors like medicine, agriculture, food and paper industries, laundries etc,due to their wide applications in various processes. The enzymes which has studied in this work are also used in various sectors as they show activity at variable temperature and pH.

Future research should focus on confirming the Km value for all three enzyme synthesis, purifying and deeply characterising all three enzymes generated and optimising the growing conditions of halophilic isolates for future applications in various industries.

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