International Journal of Life science & Pharma Research

# PURIFICATION OF B-KETOTHIOLASE FROM HALOPHILIC ARCHAEA HALOARCULA SP.1 ISOLATED FROM BHAVNAGAR COAST, GUJARAT, INDIA.

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## ABSTRACT

Members of the Archaeal family have been determined to accumulate Poly (3-hydroxybutyrate) (PHB) during their growth. A total of 13 extremely Halophilic Archaeal isolates designated as NPW-1 to NPW-13 were capable of accumulating large amounts of PHB. Out of which best four isolates were selected from enzyme assay. Since measurements of enzyme activities related to Archaeal PHB biosynthesis have never been achieved, we investigated the first enzyme of PHB biosynthesis in *Haloarcula* sp.1 i.e., β-ketothiolase. Crude extracts of strain cultivated under accumulating conditions showed maximum β-ketothiolase activity. β-ketothiolase was partially purified by ammonium sulfate fractionation and highest activity was obtained in 60% saturation fraction. This is the first description of an archaebacterial β-ketothiolase . Silver staining of the purified enzyme (fraction 8) with SDS – PAGE showed that enzyme subunited molecular weight putatively identified was 45 kDa.

**Keywords:** Extremely halophilic Archaea, β-ketothiolase, Poly-β-hydroxybutyrate (PHB), hypersaline environments

## INTRODUCTION

The Archaea (Woese *et al*., 1990) constitute a separate domain of possibly ancient organisms that exist under extreme conditions such as high salinity, temperature etc. Investigations on Archaeal genomes and enzymology are of interest because they may provide information helpful in understanding the evolution of early life (Woese, 2000). The major role attributed by enzyme β-ketothiolase is in fatty acid degradation while the primary role of enzyme is in biosynthesis PHB which is extensively discussed. Thiolases have been purified throughout the phylogenetic scale (from higher eukaryotes to yeast to prokaryotes) and genes encoding for them also have been cloned (Antonenkov *et al*., 2000a, b). Information concerning enzyme and its purification from halophilic Archaea is very sparse, only the unique case reported so far from extremely halophilic Archaea is enzyme acetoacetyl – CoA thiolases. As very little has been done so far on the halophilic enzyme participating in PHB biosynthesis and to extend further knowledge of thiolase, we describe with purification of β-ketothiolase from extremely halophilic *Archaea* which can be useful for its characterization and also in increasing the bioplastics (Poly Hydroxybutyrate) production (Senior and Dawes, 1973; Kyriakidis *et al*., 2005).

## MATERIALS AND METHODS

### *Collection of samples*

Samples (Water and sediment) were collected aseptically in sterile glass bottles and the plastic bags from salt pans at Newport and Nari, Bhavnagar, Gujarat, India (Latitude 21º45’ N and Longitude 72º14’ E and 22º10’ N and 72º15 E).

***Enrichment of Halophilic Archaea***

Halophilic Archaea were enriched in Tryptone yeast extract salt (TYES) (Krieg and Holt, 1984), Mullakhan and Larsen, 1975 (M & L) and Larsen (Larsen, 1981) media containing 100µg/ml each of penicillin G, erythromycin and cycloheximide to inhibit growth of bacteria and fungi and incubated at 37 °C for 15 – 20 days (Oren and Litchfield, 1999). From enriched halophilic Archaeal broth organisms were streaked on respective agar plate for the purpose of isolation of pure culture. A total of 13 isolates were obtained designated as NPW-1 to NPW -13 and preserved. All the isolates were preliminary screened for PHB production. Potent four PHB producers were used further to study enzyme activity.

***Growth and kinetics of β – ketothiolase Producers***

The maximum four PHB producing isolates were inoculated in to 100 ml of TYES medium and incubated at 37 °C on shaker at 180 rpm. The cells were harvested by centrifugation at 10,000 rpm for 15 min after 14 days incubation. The cells were lysed in 2% sodium hypochlorite solution and again centrifuged. The supernatant was then assayed for the presence of β – ketothiolase as mentioned below (Nishimura *et al*., 1978; Satoh *et al*., 2002).

***β – ketothiolase assay (Senior and Dawes, 1973)***

β – ketothiolase activity was assayed by the thiolysis of acetoacetyl – CoA. The assay mixture (a total volume of 1 ml) contained 100mM Tris – HCl buffer (pH 8.1) (750 µl), 60mM $MgCl_2$ (100 µl), 0.05mM acetoacetyl – CoA (10 µl), 0.05mM CoA (30 µl) and β – ketothiolase (10µl). The culture supernatant containing the enzyme was added after preincubation of the reaction mixture at 25°C for 2 min. The decrease in acetoacetyl CoA was then measured spectrophotometrically at 303nm at 30°C using a millimolar extinction coefficient of 12.9$mM^{-1}cm^{-1}$.One unit of β – ketothiolase was defined as the amount of enzyme that catalyzes the cleavage (conversion) of 1 µmol of acetoacetyl CoA in 1 min. The absorption at 303nm is due to the chelation of magnesium to the enolate form of acetoacetyl – CoA (Stern, 1956).

***Identification of isolate producing maximum β – ketothiolase enzyme activity***

One of the isolate which showed maximum β – ketothiolase enzyme activity had been identified by its molecular characterization i.e., 16S rDNA partial sequencing (Xcelris Labs., Ahmedabad, Gujarat, India).

***Optimization of Media for β – ketothiolase production***

Among the four isolates maximum enzyme was produced from *Haloarcula* sp. 1 which was further selected for optimization. Various parameters used for optimization were pH, temperature, salt, Inoculums sizes, Carbon source, Nitrogen Sources and phosphate as substrates (Data Not Shown).

***Partial Purification of β – ketothiolase Enzyme***

Among the four isolates maximum enzyme was produced from *Haloarcula* sp. 1 which was further selected for optimization and the purification of the β – ketothiolase. $10^8$ cells / ml of *Haloarcula* sp. 1were inoculated into 100ml TYES medium containing glucose (5 g/l) and ammonium chloride (0.02 g/l) as carbon and nitrogen in ratio 3:1 with all the optimized conditions as mentioned above (Data not Shown). Eight days old culture was reinoculated in 1L TYES medium under similar conditions and incubated at 37°C for 8 days. All the steps were carried out at 20 - 24°C unless otherwise indicated. Step – 1 *Crude extraction*: - 1L culture was centrifuged and pellets were collected at 10,000 rpm for 15 min at 4°C (Eppendroff 5840R). About 40g of wet cells of *Haloarcula* sp. 1 were suspended in a 200ml breakage buffer [50Mm Tris – HCl, pH 7.0, 2M KCl, 5mM 2 – mercaptoethanol, 1mM phenylmethylsulfonyl fluoride (PMSF)], and disrupted by sonic oscillation using TOSHCON sonicator for (10 min with 3s of pulse and 2s of pulse off time) 10 cycles of 15 sec on and 45 s off (Liu *et al*., 2007). The cellular lysate was centrifuged at 10,000 rpm for 15 min and the supernatant was used as "crude extract" for enzyme purification (Liu *et al*., 2002). Step – 2 *Ammonium sulphate*: - The cell extract was saturated 40 – 60% with ammonium sulphate AR grade with slow stirring on ice for 1 – 2 h and the precipitated proteins were removed by centrifuging at 10,000 rpm for 20 – 30 min at 0°C. The supernatant was dialyzed for 24 h against two changes of 5L portions

of 10mM Tris – HCl buffer pH 7.3 plus 1mM dithiothretiol. Step – 3 *DEAE – Cellulose chromatography*: - The dialyzed crude enzyme solution was loaded on DEAE – Cellulose column (28 x 2.6 cm) previously equilibrated with buffer. The enzyme then eluted at 4°C by running a linear gradient of KCl (0 – 300mM) in 2 × 300 ml of 10mM Tris – HCl buffer, pH 7.3, plus 1Mm dithiothreitol at a speed of 1ml/ min. Eluted fractions were assayed for protein and enzymic activity. Step – 4 *Sephadex G – 200 chromatography*: - The active fractions obtained from the previous step were combined and loaded on Sephadex column (2.5 x 5 cm) previously equilibrated with 10mM Tris – HCl, pH 7, plus 0.5mM dithiothreitol. The column was washed with 50ml of the same buffer and the enzyme activity was eluted by stepwise elution with linear gradient of KCl (0 – 300mM) 2 × 300 ml of 10mM Tris – HCl buffer, pH 7.3, plus 1Mm dithiothretiol at a speed of 1ml/min. Eluted fractions were assayed for protein and enzymic activity.

***SDS Polyacrylamide gel electrophoresis (SDS – PAGE) (Laemmli, 1970)***

SDS PAGE was performed on 12 % separating and 5 % stacking polyacrylamide gels at 25˚C using Miniprotean II (Biorad) apparatus. The gels loaded with enzymes samples and molecular markers (Bangalore Genei) were run at 25 / 50 V till the dye front reached the end of the separating gel. The proteins bands were visualized by silver staining (Morrisey, 1981).

## RESULTS AND DISCUSSION

***Identification of isolate producing maximum β – ketothiolase enzyme activity***

Results of 16S rDNA partial sequencing (642 bp) of the strain NPW-9 showed maximum sequence identity (100%) with the complete sequence of *Haloarcula* sp. AB19 (GenBank Accession No. DQ471854.1) (Fig. 1). Thus, the isolate NPW-9 is affiliated to *Haloarcula* species and hence in the present study it is referred to as *Haloarcula* sp.1 as referred to previously.

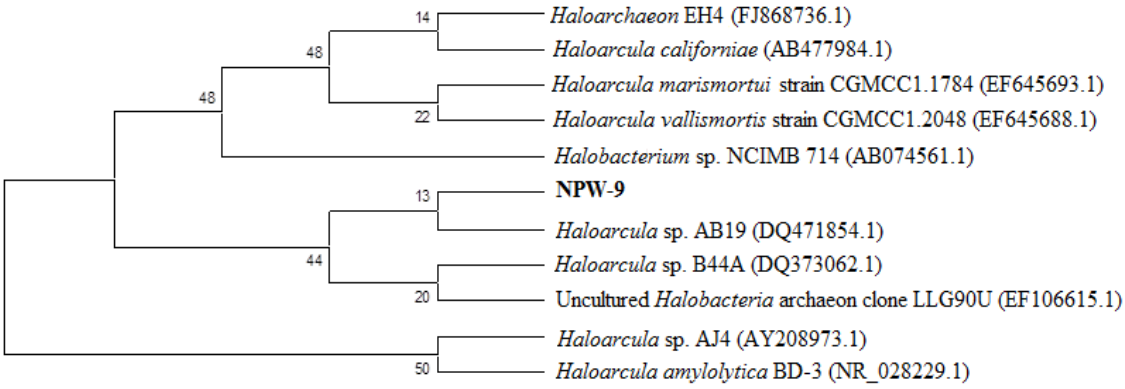



**Figure 1**
***Phylogenetic tree of isolate NPW-9 (using neighbor-joining method)***

Table 1summarizes the purification procedure of the enzyme. Following all the steps i.e. ammonium sulfate precipitation, dialysis and column chromatography, 39.3 fold purification of β-ketothiolase with an overall recovery of 12.02%, and specific activity 1.181 units/mg was obtained. During each purification step, specific activity and fold purification increased, while enzyme activity and % yield decreased. As shown in Fig. 1, fraction 8 showed highest activity and was used for purification and determination of its molecular weight on SDS PAGE. The purified β-ketothiolase was analyzed by SDS PAGE. Silver staining of the purified enzyme (fraction 8) with SDS – PAGE showed that enzyme subunit molecular weight putatively identified was 45 kDa (Fig. 2) (Kyriakidis *et al*., 2005). SDS PAGE with crude enzyme preparations showed multiple bands with one of the bands at the same position as in purified preparation

indicated the presence of β-ketothiolase also in the crude enzyme preparation.

## CONCLUSION

In the present work the purification of a β – ketothiolase of *Haloarcula* sp. 1, the first enzyme of the biosynthetic pathway of PHB has been described from a halophilic archaeon *Haloarcula* sp. 1. This is the first report on purified halophilic β – ketothiolase that acts at high salt concentrations. These thiolases exhibit not only an extreme salt requirement but also has unique kinetic properties that differ significantly from *Bacteria* and *eukarya*. It is possible that there is a special thiolytic mechanism in haloarchaea. The results are supported by Kyriakidis *et al*., 2005 who obtained a single band of 45.5 KDa molecular mass on SDS – PAGE with purification of 390 fold and 7% recovery. Papoutsakis *et al*., 1988 have performed denaturing gel electrophoresis and reported that the enzyme obtained from *Zoogloea ramigera* and *Clostridium acetobutylicum* ATCC 824 showed molecular weight as determined by PAGE to be 44 kDa.

**Table 1**
***Purification of β-ketothiolase from Haloarcula sp. 1***

| Purification step | Volume (ml) | Protein (mg/ml) | Total Protein (mg) | Enzyme activity (units) | Specific activity (Units/mg) | Purification Fold | Recovery (%) |
|---|---|---|---|---|---|---|---|
| Crude extract | 600 | 29.23 | 17542.19 | 532 | 0.030 | 1 | 100 |
| $(NH_4)_2SO_4$ fractionation | 30 | 24.88 | 746.46 | 159 | 0.213 | 7.1 | 29.88 |
| DEAE – cellulose column | 10 | 19.63 | 196.3 | 89.73 | 0.457 | 15.24 | 16.86 |
| Sephadex G – 200 column | 5 | 10.83 | 54.15 | 63.97 | 1.181 | 39.3 | 12.02 |

**Figure 1**
***Elution profile of β – ketothiolase showing enzyme activity, protein Content and specific activity of Haloarcula sp. 1***

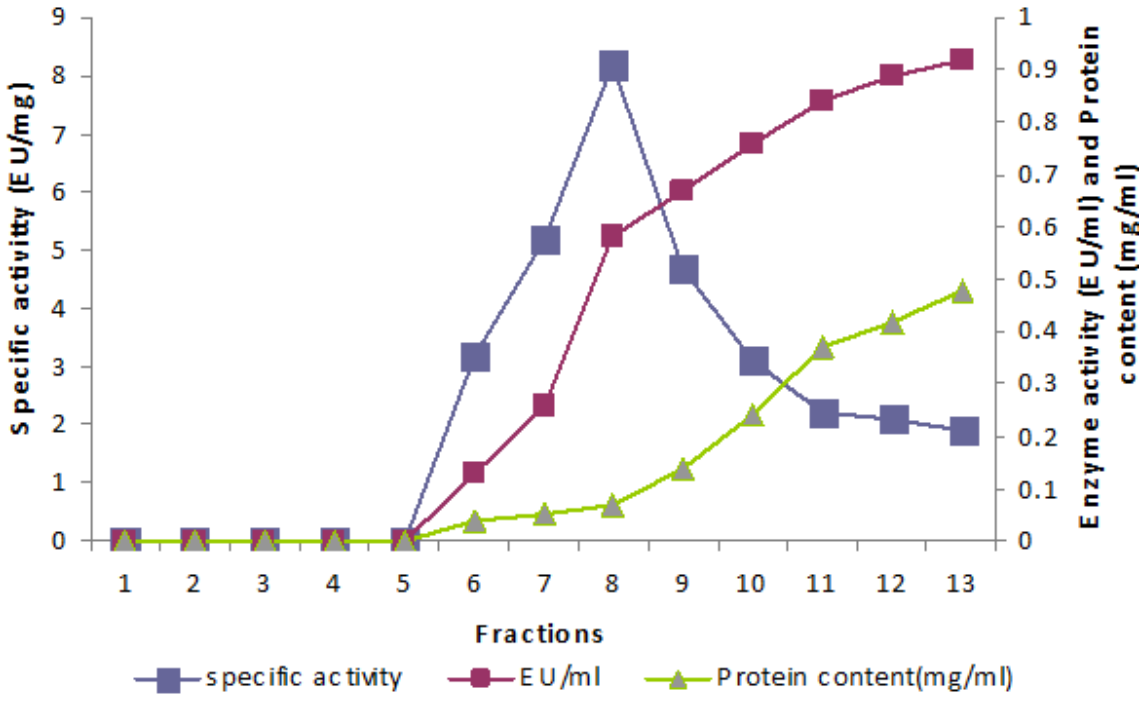
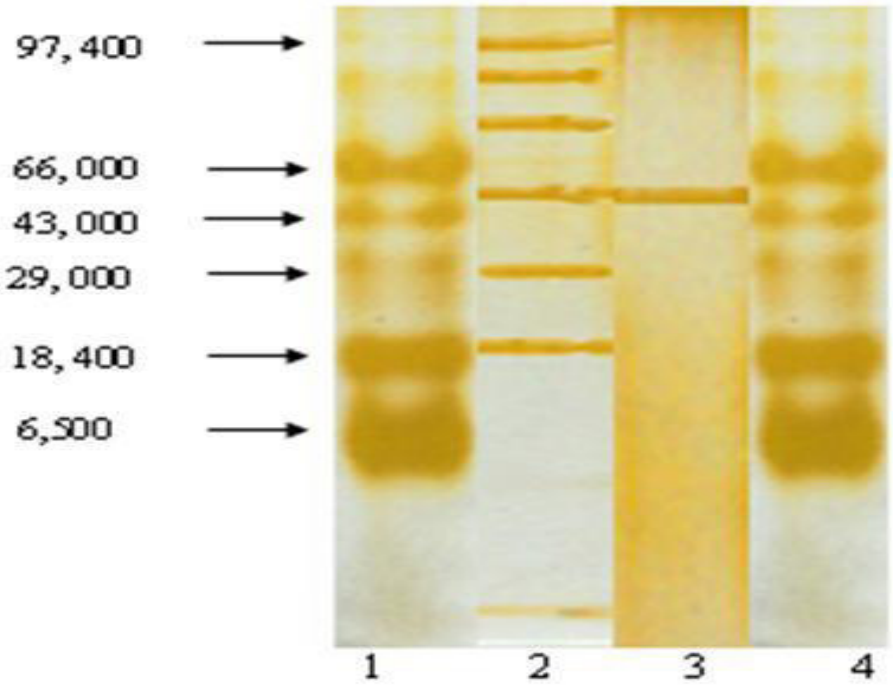


**Figure 2**
***SDS PAGE: Lane 1: Molecular Marker, Lane 2: crude extract, Lane 3: purified enzyme Lane 4: Molecular Marker***

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