

**STUDIES ON MODERATELY HALOPHILIC  
*MARINOBACTER* SP. EMB8 AND ITS SALT AND  
SOLVENT STABLE  $\alpha$ -AMYLASE**

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SOLVENT STABLE  $\alpha$ -AMYLASE**

*by*

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**DEPARTMENT OF CHEMISTRY**

*Submitted*

*in fulfillment of the requirements of the Degree of*

**DOCTOR OF PHILOSOPHY**

*to the*



**INDIAN INSTITUTE OF TECHNOLOGY DELHI**

**FEBRUARY, 2014**

*Dedicated to my Grandfather*

# CERTIFICATE

This is to certify that the thesis entitled “Studies on moderately halophilic *Marinobacter* sp. EMB8 and its salt and solvent stable  $\alpha$ -amylase” being submitted by **Mr. Sumit Kumar** to the Indian Institute of Technology Delhi for the award of the degree of *Doctor of Philosophy* in Chemistry is a record of bonafide research work carried out by him. Mr. Sumit Kumar has worked under my guidance and supervision, and has fulfilled the requirements for the submission of the thesis which, to my knowledge, has reached the requisite standard.

The results contained in this dissertation have not been submitted in part or full to any other University or Institute for the award of any degree or diploma.

February, 2014

Dr. S. K. Khare  
Professor of Biochemistry  
Department of Chemistry  
Indian Institute of Technology Delhi

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**Sumit Kumar**

New Delhi

# Abstract

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Halophiles are the exotic group of extremophiles inhabiting saline and hypersaline environments. Sustenance under such conditions necessitates the cells to acquire specific osmolytes or accumulate equivalent salt in cytoplasm, for preventing exosmosis and dehydration. The halophilic enzymes have specific features for maintaining their structure and carry out catalysis in the presence of high cytoplasmic salt or osmolytes. This makes them unique and different than non-halophilic proteins. There is a great deal of interest in halophilic enzymes, to exploit them for performing reaction/ efficient catalysis in saline and solvent medium.

The thesis presents study of a novel  $\alpha$ -amylase from a halophilic *Marinobacter* sp. EMB8 isolate. It encompasses study of halophilic microbial diversity in Indian saline habitats and profiling of their hydrolases. The *Marinobacter* sp. selected in this study has been characterized in detail. Its  $\alpha$ -amylase has been purified to homogeneity, characterized for kinetic parameters, stability at secondary and tertiary structure in salt/solvents and molecular characteristics.

The application of  $\alpha$ -amylase has been amply demonstrated in nano immobilized form. The highlight of the work is that  $\alpha$ -amylase generates industrially desirable maltotetraose and maltotriose rich maltooligosachharides from starch hydrolysis in presence of solvents by exploiting its salt/ solvent stable nature.

Eighty eight halophilic bacterial strains exhibiting good hydrolases (amylases, lipases and proteases) activity were isolated. The potential isolates among these were

characterized by morphological, physiological, biochemical and 16S rDNA analysis. These were related to *Marinobacter*, *Virgibacillus*, *Halobacillus*, *Geomicrobium*, *Chromohalobacter*, *Oceanobacillus*, *Bacillus* and *Halomonas* genera. The 16S rDNA sequence of isolates have been submitted in GenBank, National Center for Biotechnology Information (NCBI), USA and assigned corresponding accession numbers. Their essential salt requirement ranged from 3-20% (w/v), indicating them to fall under moderately halophilic category. They showed diverse metabolic activities and carbon source utilization on Biolog study. Preliminary characterization of crude hydrolases established them to be active and stable under polyextreme conditions of high salt, pH, temperature and presence of organic solvents.

$\alpha$ -Amylases are lesser worked out enzyme among halophiles. Hence, amylase producers among the isolates were pursued for further study. The *Marinobacter* sp. EMB8 (GU059908) secreted maximum amylase as observed by the zone of hydrolysis on starch agar plate. It also produced significant amount of amylase in the broth culture. Preliminary studies revealed its amylase to be stable in the presence of salt and various hydrophobic solvents. In view of the above, this strain was investigated further. The culture has also been deposited in Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India.

The halophiles suffer from the drawback of low level of enzyme production, which often limits their applications as source for industrial enzymes. The optimization of culture conditions for achieving maximum amylase production was carried out.

Among the carbon sources, starch, dextrin and maltose supported growth and amylase production. Starch was found to be the best carbon source at 5% (w/v) concentration. Glucose acted as catabolic repressor for amylase production and no activity was detected in the medium with glucose as the sole carbon source.

Casein enzyme hydrolysate (1%, w/v) was found to be the best nitrogen source for amylase production. Salt proved critical for amylase production and maximum production was attained at 5% (w/v) NaCl. Under finally optimized conditions in the medium (g/L): starch, 50.0; casein enzyme hydrolysate, 10.0; K<sub>2</sub>HPO<sub>4</sub>, 0.87; MgSO<sub>4</sub>.7H<sub>2</sub>O, 6.2; NaCl, 50.0; adjusted to pH 7.0, while seeded with 1% (v/v) inoculum and incubated at 35 °C and 200 rpm, resulted into production of 48.0 IU amylase/mL. Optimization led to about 12 fold increase in the production.

The  $\alpha$ -amylase was purified to homogeneity by ultrafiltration, diethylaminoethyl-cellulose (DEAE-cellulose) and Sephadex G-75 column chromatography with 52% yield and 76 fold purification. It was a monomeric protein of 72 kDa. The  $\alpha$ -amylase had many novel features *viz.* stability up to 20% NaCl, 80 °C temperature, 6.0-11.0 pH range and in a wide range of organic solvents at high concentrations.

The enzyme exhibited broad substrate specificity. It was active towards dextrin, amylose, amylopectin and glycogen, however soluble starch was found to be the best substrate. No activity was detected on  $\alpha$ -cyclodextrin,  $\beta$ -cyclodextrin and pullulan indicating it to be an endolytic enzyme. The  $K_m$  and  $V_{max}$  of the  $\alpha$ -amylase towards soluble starch were 4.6 mg/mL and 1.3 mg/min/mL respectively. It exhibited temperature optimum at 45 °C and a pH optimum at 7.0.

The novel feature was its significant thermal stability, which was much better as compared to other known halophilic amylases. It had a half life of 80 minutes at 80 °C. The activity was unaffected by  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  ions. Ethylenediaminetetraacetic acid (EDTA) at 5mM concentration caused about 68% loss in activity. The results clearly established the necessity of salt for catalytic activity. No activity was observed in absence of NaCl/ KCl. The optimum activity was recorded at 1% (w/v) NaCl. Since *Marinobacter* sp. is a moderate halophile, its enzymes possibly require less salt concentration for optimum catalysis.

Among the additives,  $\alpha$ -amylase was not affected by phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT) and  $\beta$ -mercaptoethanol. However, loss in activity with PCMB indicated the involvement of sulfhydryl group(s). It was relatively stable in presence of surfactants like sodium dodecyl sulphate (SDS), Tween 80 and Triton X-100 and retained more than 75% activity after 1 h exposure at 0.1% (w/v) concentration.

$\alpha$ -Amylase in the present study was stable in 25% (v/v) concentration of dichloromethane, benzene, toluene, hexane, cyclohexane and decane up to 24 h without any loss in activity. Solvent stability has been observed as a generic and unique trait among the halophilic enzymes in recent years. This is attributed to the fact that they work at high salt surroundings which is akin to low water medium.

The structural study of the purified enzyme was carried out by circular dichroism and intrinsic fluorescence spectroscopy. Structural changes recorded in presence of varying amount of NaCl (5-20%, w/v) exhibited increase in negative ellipticity as a function of salt, confirming that salt stabilizes the protein with increase in secondary

structure. The data of intrinsic and extrinsic fluorescence (using 1-anilinonaphthalene-8-sulfonate (ANS) as probe) supported the similar role of salt. Guanidine hydrochloride and pH denaturation indicated the molten globule state at pH 4.0.

The study also covers the results pertaining to the bioinformatic analysis of the  $\alpha$ -amylase gene. N-terminal amino acid sequence of the purified  $\alpha$ -amylase was experimentally determined. Based on this sequence, NCBI database was searched for its homologues using the BLASTP program. The search revealed the relatedness to *Pseudoalteromonas* sp. amylase. The N-terminal signature sequence “FVHLFEW” was found as conserved in many eukaryotic and bacterial  $\alpha$ -amylases. Structure-function relationship and evolutionary lineage of halophilic amylases was established. Three dimensional structure of *Marinobacter algicola*  $\alpha$ -amylase having N-terminal signature sequence was deduced using I-Tasser server.

The application of *Marinobacter* sp.  $\alpha$ -amylase in maltooligosachharide (MOS) synthesis was explored. The  $\alpha$ -amylase efficiently hydrolyzed starch into MOS rich in maltotriose and maltotetraose. This is a very desirable feature, as predominantly maltotriose and maltotetraose rich MOS are considered better in bakery applications. Since, these prevent migration of moisture from starch granules, reduce retrogradation and interfere with starch-gluten interaction.

About 72% starch hydrolysis was attained in 4 h. Thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) analysis of soluble starch hydrolysis revealed that maltotetraose, maltotriose and maltose were major products.

Quantification of MOS species by HPLC showed 40% maltotetraose formation in 30 minutes which gradually decreases to 21% in 2 h and 5% in 4 h, with concomitant increase in maltose from 28% in 30 minutes to 62% in 4 h. This indicated that initially formed maltotetraose could be hydrolyzed into maltose. However, maltotriose concentration remains constant at 30-33% from beginning to 4 h, confirming *Marinobacter*  $\alpha$ -amylase to be a maltotriose forming enzyme. The starch hydrolysis carried out in presence of 10% (v/v) ethanol led to three times increase in maltotetraose concentration. Thus advantage of solvent stability can be exploited to enhance maltotetraose yield.

In order to facilitate further use of the  $\alpha$ -amylase, it was immobilized on 3-aminopropyl functionalized silica nanoparticles by using glutaraldehyde as cross-linking agent. Optimization of various parameters resulted in 96% immobilization efficiency. Optimized conditions for immobilization were 0.5% (v/v) glutaraldehyde, 0.5% (w/v) starch, 90 IU  $\alpha$ -amylase and 15 mg nanoparticle concentration. The enzymatic properties mostly remained unaltered. Immobilized amylase retained 75% of its activity after 5<sup>th</sup> repeated use. Starch hydrolyzing efficiency of immobilized enzyme was slightly better as compared to the native enzyme.

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