Chapter 2

Cloning, Expression, Purification and Characterization of HutP from 

*Geobacillus Thermodenitrificans*

2.1. Introduction

*Geobacillus thermodenitrificans* is a thermophilic bacterium. It was first isolated from oilfield in Dagang (Northern China) at a depth of 2000 m and at a temperature of 73°C. The complete genome sequence consists of 3550319-bp chromosomes and 57,693-bp plasmids (Feng *et al.*, 2007).

*Geobacillus thermodenitrificans* have many genes involving in various activities such as transporters of nutrient uptake, detoxification, respiration system including an aerobic branch comprising five terminal oxidases and an anaerobic branch comprising a complete denitrification pathway for immediate response to dissolved oxygen fluctuation. Recent literature studies also confirmed the availability of *hutp* gene in *Geobacillus thermodenitrificans*. From sequence analysis, the *hutP* gene in *Geobacillus thermodenitrificans* consists of 149 amino acids, and about 60% sequence similarity with the earlier reported structures of HutP from mesophilic organism *Bacillus subtilis*. Generally thermophilic organism has higher thermal stability than mesophilic bacteria. Reports indicate that the thermophilic bacteria have more number of ion pairs, hydrogen bonds and aromatic interactions in its structural arrangements.

Based on the above aspects, we plan to determine the crystal structures of HutP from thermophilic bacterium *Geobacillus thermodenitrificans*. 
2.2. Subcloning of hutP gene

The hut gene from *Geobacillus thermodenitrificans* was chemically synthesized with engineered His-tag and thrombin cleavage sites at N-terminal region. The synthesized sequence was cloned into pCR 2.1 vector. The cloned gene was subcloned using TA cloning methods.

2.3. Digestion reaction of hutP gene

The cloned gene was digested with *NdeI*, *BamHI* (NEB, UK) restriction enzymes. Digestion of *hutP* gene with *NdeI* (1 µl=20 U), *BamHI* (1 µl=100 U) enzymes were carried out using the reaction mixture as given in Table (Table 2.1). The reaction was carried out for 5 hours at 37°C. After the reaction time, reaction mixture was loaded onto the 5% agarose gel electrophoresis. The gel was stained using ethidium bromide dye. The digested product of *hutP* gene (450 bp) from pCR 2.1 vector was observed in the gel (Fig. 2.1).

2.4. Gel extraction

The digested *hutP* gene was purified by gel extraction method using QIAGEN kits. The agarose gel was viewed through UV source; the digested region was marked and separated into slices. The slices were weighed and collected into 2 ml eppendorf tubes. Three volumes of QC buffer was added to tube containing gel slices and incubated at 50°C until the gel was completely dissolved. One volume of isopropanol was added and mixed thoroughly. The samples were transferred to spin column and centrifuged at about 8500 rpm for a minute. The supernatant was discarded; pellet was mixed with 500 µl of QC buffer and centrifuged at 8500 rpm. Then 750 µl of PE buffer was added to pellet and centrifuged for a minute at 8500 rpm. The column was transferred to a fresh tube and allowed to dry in room temperature.
Table 2.1. Digestion reaction composition

<table>
<thead>
<tr>
<th>Composition</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template</td>
<td>7.00</td>
</tr>
<tr>
<td>Buffer</td>
<td>5.00</td>
</tr>
<tr>
<td>H₂O</td>
<td>36.25</td>
</tr>
<tr>
<td><em>NdeI</em></td>
<td>1.50</td>
</tr>
<tr>
<td><em>BamHI</em></td>
<td>0.25</td>
</tr>
<tr>
<td>Total</td>
<td>50.00</td>
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</table>

Fig. 2.1. Agarose gel pattern of digested *hup* gene. Digested product 3µl (lane 1), DNA Ladder (lane 2), Digested product 2µl (lane 4). The arrow mark on right side indicates the Digested product of *hup* gene around 450 bp ranges.
Finally 50 µl of EB buffer were added and the DNA was retrieved from centrifugation at 8000 rpm and the concentration was measured using Nanodrop spectrometer (Thermo Scientific). The purity of the DNA was once again confirmed by 6% agarose gel.

2.5. Ligation of hutP

The digested product was used for ligation. The pHCE vector was digested with NdeI, BamHI enzyme taken forward for ligation with hutp gene. The reaction mixture composition is given in Table 2.2. The reaction was carried out for overnight at room temperature. The ligation of hutp with pHCE vector was analyzed by agarose gel electrophoresis method (Fig. 2.2). The ligated DNA again digested with NdeI, BamHI restriction enzymes to reconfirm the results. The sub-cloning of hutp gene from Geobacillus thermodenitrificans was successfully completed.

2.6. DNA sequencing

The cloned gene was further confirmed by sequencing the DNA using capillary electrophoresis method (Applied Biosystem 3100 HITACHI). Prior to the sequencing, purity and concentration of the DNA plasmids were measured. The primers of backbone (pHCE vector) were chosen to amplify the inserted DNA plasmids. The PCR product was loaded on to agarose gel to confirm the amplification. Amplified DNA was purified using the method provided in QIAGEN plasmid purification for DNA sequencing. Purified plasmids were applied to cycle sequencing followed by capillary electrophoresis. The resulted spectrum was analyzed using Finchtv software (Fig. 2.3).
Table 2.2. Ligation reaction composition

<table>
<thead>
<tr>
<th>Composition</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHCE Vector</td>
<td>1.50</td>
</tr>
<tr>
<td>Buffer</td>
<td>2.00</td>
</tr>
<tr>
<td>DNA</td>
<td>15.00</td>
</tr>
<tr>
<td>H₂O</td>
<td>1.25</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20.00</strong></td>
</tr>
</tbody>
</table>

Fig. 2.2. Ligated product of *hutP* gene with pHCE vector. The ligated product was loaded on gel 2 µl (lane 1) and 3 µl (lane 2). The ligated gene was indicated by an arrow mark.
Fig. 2.3. DNA sequencing of hutP gene
2.7. Plasmid preparation

Ligated product (7 µl) was added to DH5α cells followed by incubation for 20 minutes in ice-cold condition. The sample was given a heat-shock treatment at 42°C for a minute and brought back to the ice-cold condition. Added 200 µl of LB media to the reaction mixture and was incubated at 37°C with constant agitation for an hour and then the cells were transformed to LB plates containing the antibiotic, ampicillin (50 µg/ml). The plates were incubated at 37°C shaker overnight. The good grown colonies were observed on next day and the plate was stored at 4°C. Single colony was selected from plate and used for inoculation in the presence of ampicillin (50 µg/ml) containing culture tubes followed by 5 ml of LB medium. The tube was incubated overnight at 37°C in water bath.

The DNA isolation was carried out using Qiagen miniprep kits (QIAGEN)

1. Cultures were centrifuged and pellet was collected.
2. Pellet was mixed with 250 µl of P1 buffer (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 100 ug/mL RNase A) and sample was mixed thoroughly.
3. 250 µl of P2 buffer (200 mM NaOH, 1% SDS) was added (mixed properly by inverting the tubes).
4. The 350 µl of N3 buffer (4.2 M Gu-HCl, 0.9 M potassium acetate, pH 4.8) added to the tube.
5. Centrifuged at 13000 rpm for 10 minutes and the supernatant added onto the column, centrifuged the sample for a minute at 8000 rpm.
6. 500 µl of PB buffer (5 M Gu-HCl, 30% isopropanol) added to the pellet and the sample was centrifuged around 8000 rpm for a minute.
7. 750 µl of PE (10 mM Tris-HCl pH 7.5, 80% ethanol) buffer was added to the column and centrifuged.
8. The supernatant was removed and column dried in room temperature, the 50 µl of EB (10mM Tris-HCl, pH 8.5) buffer was added, the DNA sample collected by centrifugation at 8000 rpm.

The concentration of plasmid DNA was measured using Nanodrop spectrometer.
2.8. Transformation in expression vector

The transformation was carried out as described in Section 2.7. Whereas, the BL21 (DE3) cells were used for expression instead of DH5α cells. The 0.5 µl of construct (hutp-pHCE) was added to the expression vector followed by LB medium with ampicillin and incubated at 37°C. Colonies appeared on plates after overnight incubation (Fig. 2.4).

2.9. Protein expression

To clarify the protein expression, we used small-scale preparation before going to large-scale production. The colonies from BL21 (DE3) expression cells were inoculated at 37°C for overnight in LB medium in the presence of ampicillin without any induction (IPTG). The well-grown culture was harvested by centrifugation at 8000 rpm for 15 minutes. The pellet was lysed with buffer containing 50 mM Tris-HCl (pH8), 500 mM NaCl, 5% glycerol and 2 mM β-mercaptoethanol. The completely dissolved sample was sonicated in ice-cold condition for a minute and repeated about 10 times with regular interval of 1 minute.

After sonication, the total lysate was centrifuged at 13000 rpm, the supernatant and pellet were collected for SDS PAGE analysis. The sample was loaded with different concentration to examine the expression level on to the 15% SDS-PAGE, along with the pre-stained standard marker (Bio-rad). The gel was stained using Coomassie Brilliant Blue (CBB) dye and destained. Good expression was observed near 16 kDa region, confirming the expression of HutP protein (Fig. 2.5).
**Fig. 2.4.** Transformation in BL21 (DE3) cells

**Fig. 2.5.** 15% SDS-PAGE of expressed HutP protein. The standard markers 10-250 kDa are loaded on lane 1. The supernatant and the pellets were loaded alternatively in different volumes from lane 2 to 13. The expressed HutP was indicated by arrow.
2.10. Purification of HutP

After confirming the small-scale expression of HutP, we prepared 2 litres LB culture as per the protocol mentioned in Section 2.9; the expression was analyzed using SDS-PAGE. Contamination was also observed at higher molecular range along with the Hutp found at 16 kDa range.

2.11. Purification of HutP using Ni-NTA column

The presence of His-tag in the protein helped us in choosing Ni-NTA column for the first step of purification.

Buffers used for Ni-NTA Purification

<table>
<thead>
<tr>
<th>Buffer A</th>
<th>Buffer B</th>
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<tbody>
<tr>
<td>50 mM Tris-HCl pH=8.0</td>
<td>50 mM Tris-HCl pH=8.0</td>
</tr>
<tr>
<td>500 mM NaCl</td>
<td>500 mM NaCl</td>
</tr>
<tr>
<td>5% glycerol</td>
<td>5% glycerol</td>
</tr>
<tr>
<td>2 mM β-mercaptoethanol</td>
<td>2 mM β-mercaptoethanol</td>
</tr>
<tr>
<td></td>
<td>1 M Imidazole</td>
</tr>
</tbody>
</table>

The column was washed twice and equilibrated using buffer A. After equilibration sample was loaded on to the column and the flow through was collected using fraction collector. The bound sample was eluted by imidazole containing buffer (Buffer B) through step-gradient method (5 mM, 25 mM, 50 mM, 100 mM, 300 mM and 500 mM). The eluted fractions were loaded onto 15 % SDS-PAGE (Fig. 2.6).
Fig. 2.6. 15 % SDS of Ni-NTA elution. Lane 1 & 2: markers; The eluted fractions are loaded in different concentration as follows: 5 mM elution (lane 3, 4); 25 mM elution (lane 5, 6); 50 mM elution (lane 7, 8); 100 mM (lane 9, 10); 300 mM elution (lane 11, 12, 13, 14); 500 mM elution (lane 15, 16).

HutP was eluted at 100-500 mM of imidazole concentration with some impurities observed at lower molecular weight range. These protein fractions (100-500 mM) were pooled and dialyzed at 4°C in buffer containing 10 mM Tris-HCl, 2 mM β-mercaptoethanol to remove the imidazole. The buffer was changed every hour. After 3 hours of dialysis the sample was centrifuged at 13000 rpm, the pellet was redissolved in a buffer (50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM MgCl₂, 1 mM DTT) and allowed for overnight dialysis. While dialysis, the sample turned turbid with the formation of milky white colour precipitation. Due to the precipitate formation in above-mentioned buffer, we changed the buffer condition (20 mM Tris-HCl (pH7.5), 500 mM NaCl, 2 mM β-mercaptoethanol, 5% glycerol, 1 mM MgCl₂) and allowed
further dialysis for 3 hours. Centrifuged the solution and the protein containing supernatant solutions were collected and used for further steps.

2.12. Thrombin digestion reaction

Thrombin digestion experiment was carried out to remove the engineered His-tag, which we used for purification purpose. To estimate the amount of enzyme required for complete digestion, different concentrations (1.5 U, 2 U, and 4 U) of thrombin were added and incubated the reaction at room temperature for 2 hours. The reactions were stopped by adding PMSF (1 mM) after the incubation period. The His-tag cleavage was confirmed using SDS-PAGE (Fig. 2.7).

The SDS-PAGE showed a band shift for thrombin digestion for the samples in lane 4-9 (from 1.5 U digestions/mg). When compared with undigested (control, Lane 1 and 3) sample, the band shift around 2 kDa lower range was identified for digested samples. From the gel we confirmed the removal of His-tag. Moreover it also confirmed that 1.5 U thrombin is sufficient to cleave 1 mg of His-tag protein completely.

![Fig. 2.7. Thrombin digestion experiment. The control sample loaded on (lane 1, 3); standard marker (lane 2); 1.5 U thrombin digested sample of 2 µl (lane 4); 3 µl (lane 5); 2 U digestion 2 µl (lane 6); 3 µl (lane 7); 4 U digestion 2 µl (lane 8); 3 µl (lane 9).]
2.13. Purification of HutP by ion exchange chromatography

During Ni-NTA purification step higher molecular weight impurities were observed along His-tag after thrombin digestion. Here, we choose ion exchange chromatography method to remove contamination and His-tag from the sample.

The prepacked HiTrap QFF column was washed and equilibrated with the buffer containing 20 mM HEPES (pH6.9), 2 mM β-mercaptoethonal, 1 mM MgCl₂. The thrombin digested sample was loaded onto the column and eluted by salt gradient method from 150 mM to 1 M NaCl concentration. The flow through was also collected along with gradient fractions for further analysis. Interestingly, protein was eluted at flow through stage itself. The presence of NaCl in protein containing buffer was responsible for the elution. The cleavage of His-tag was eluted at higher gradient elution (Fig. 2.8). The final purified HutP sample was concentrated; the purity was confirmed by SDS PAGE (Fig. 2.9).

2.14. RNA binding assay

Initial biochemical studies by Oda (Oda et al., 2004) indicate that, HutP binds with mRNA in the presence of histidine availability.

Later on the biochemical and crystallographic studies of HutP and its various complex structures from *Bacillus subtilis* reveal that, the protein binds with mRNA not only the presence of ligand but also metal ions required (Kumarevel, Mizuno and Kumar, 2005).

The gel mobility shift assay was performed to clarify the ability of HutP-RNA interactions. The 21mer (5’-UUUAGUUUAGUUUUAGUU-3’) RNA was purchased (Fasmac, Japan) and used for the binding studies.
Fig. 2.8. Ion exchange elution pattern. The markers are loaded in (lane 1, 3); flow through 2 µl (lane 4); 3 µl (lane 5); 150 mM fraction (lane 6,7) 200 mM fraction (lane 8, 9,10); 300 mm elution (lane 11, 12) 500 mM fraction (lane 13, 14) and 1M fractions (lane 15, 16).

Fig. 2.9. Purified form of HutP. Standard marker loaded on lane 1; the final purified HutP was loaded on lane 2, 3 and 4. The purified HutP is indicated by an arrow.
To understand the importance of HutP, RNA, L-Histidine and MgCl₂, different complexes were prepared and their binding capacities measured.

1. HutP
2. 21mer RNA
3. HutP + L-Histidine + RNA
4. HutP + MgCl₂ + RNA
5. HutP + L-Histidine + MgCl₂ + RNA

Prepared HutP-RNA complexes were incubated in 10 mM HEPES buffer for 15 minutes. The native gel was prepared for gel mobility shift experiment (Fig. 2.10). The prepared 6% native gel was subjected to pre-run before the samples loaded at 200 V for 10 minutes at 4°C. The gel was cleaned and buffer was changed after the pre-run.

Fig. 2.10. Native gel results (6%) of HutP-RNA binding assay. HutP protein (lane 1); 21 mer RNA (lane 2); HutP+L-Histidine+RNA (lane 3); HutP+MgCl₂+RNA (lane 4); HutP+L-Histidine+ MgCl₂+RNA (lane 5 to 16) with increasing concentration of protein from 1 µl to 12. The free and shifted RNA complexes are indicated by arrows.
The samples were loaded; after the running time the gel was stained using SYBR green solution. The stained gel was visualized through Fuji film gel documentation instrument. The results show that the complex containing quaternary composition (HutP + L-Histidine + MgCl$_2$ + RNA) only undergo mobility shift, but fails when any of composition is missing (Fig. 2.10) We concluded that, HutP required L-histidine, metal ions to become active and then initiates the binding to its mRNA.