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ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF THERMOPHILIC AMINOACYLASE FROM *Geobacillus* sp. STRAIN SZN

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Abstract: A new strain from *Geobacillus* sp. was isolated from Malaysia hot spring habitat with intracellular aminoacylase activity detected at optimum performance at 60°C. *Geobacillus* strain SZN showed an optimum temperature for growth at 60 °C in pH 7.5. The 16S rRNA gene sequence of the strain showed the highest similarities to members of the genus *Geobacillus*. ProtParam analysis from Expasy tools revealed that Aminoacylase molecular weight was estimated at 41675.75 g/mol. The deduced metal binding residues are Glu139 and Glu140 and catalytic residue is Arg26.Metal binding residues and catalytic residue was notified from results of BLAST and multiple sequence alignment of *Geobacillus* SZN aminoacylase gene sequence with aminoacylase from several other species, where α-helical structure is located. The secondary structure was estimated by using SOPMA software. The data has served as a fundamental platform for development of boutique super-enzymes with desired thermostability for applications in biotechnology industries worldwide.

Keywords: *Geobacillus*sp., aminoacylase, secondary structure, thermostable enzyme, α-helix

INTRODUCTION

The search for new local thermostable aminoacylases is essential needs in global industry especially in amino acids productions industry. Enzymes from extremophiles, has becoming greater to the traditional catalysts because they can perform industrial processes even under harsh conditions, under which common proteins are completely denatured. Basically, industrial use of enzymes for production of L-amino acids has started since more than 40 years ago in Japan with the resolution of N-acetyl D, L-amino acids by immobilized aminoacylase [1] as an alternative to production of amino acids by fermentation and chemical synthesis which is faster and more specific in producing amino acids product. The industrial production of L-methionine is being practiced by immobilization of aminoacylase on to DEAE Sephadex in a packed bed reactor. Nowadays, the thermostable aminoacylases from

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thermophiles have been further modified for industrial use. Aminoacylase from *Thermococcus litoralis* have been using by Chirotech, the company of Dr Reddy's for commercial production of L-amino acids [2]. Excellent characteristics has been reported on capability of hyperthermostability aminoacylase from *Pyrococcushorikoshii* [3] and *Pyrococcusfuriosus* [4] that can withstand the catalytic activity at 90 and 100 ^{o}C, respectively.

Through this study, a new thermostable aminoacylase was isolated with greater stability at high temperatures as the criteria which needed by excellent enzymes in order to be applied in industries. In addition, the intrinsic values though molecular investigation of gene encoding thermophilic aminoacylaseand analysis of the sequence and database similarity using Basic Local Alignment Tools[5], and Biology Workbench [6] are unveiled. This study also will emphasize more about secondary structure stability and its distortion at high temperatures. Lacking of research data on secondary structure of aminoacylase from microorganism especially from high temperatures brought this study to reveal more information about factors involved in thermostable aminoacylase structure rigidity, specifically secondary structure stability and distortion when exposed at high temperatures. The stability and alteration of secondary and tertiary structures of the enzyme gives a great effect to protein folding. It plays major contributions towards its activity in extreme environments and substrate specificity [7].

Material and Methods

Isolation of Thermopile

The water samples were collected from Ulu Slim Hot spring in Perak (GPS coordinate: 3.8988° N, 101.4979° E). The samples were streaked on Luria Bertani (LB) (Difco, USA) agar plate and subsequently incubated at 60°C for 16 h (overnight). A loopful of the growth colony was transferred into 10 mL LB broth (Difco, USA) and then incubated at 50 to 90°C for 16 h. The optimum growth temperature was determined from the best bacterial growth by measuring bacteria growth at optical density 600 nm [OD_{600nm}] [8]. The media was prepared according to methods prepared by Sigma-Aldrich (Germany).

Screening for Aminoacylase Producing Thermopile

Thermophile that grow at optimum temperature were selected to screen for aminoacylase activity. The aminoacylase activity was determined *via* ninhydrin colorimetric assay [9]. A loop of bacteria colony was inoculated into LB broth (Difco, USA), Miller (1972) and incubated at 30, 40, 50, 60, 70, 80 and 90°C for 16 hours. The bacteria were collected *via*

centrifugation at 12 000 rpm and 4°C for 10 min. in order to determine the optimum growth temperature, the cell culture turbidity was measured at OD_{600nm}priorto protein extraction [8].

Preparation of Cell Extract

Aminoacylase is known as intracellular protein [10], the crude cell extract for assay was prepared from 10 mL of the culture growth at 60°C overnight in LB broth. Supernatant of the culture was removed by centrifugation at 12000 rpm and 4 °C for 10 min. The precipitated cells were washed with 50 mMTris-HCl (pH7.5). The cells were then resuspended in 10.0 mL of 50 mMTris-HCl (pH7.5) and disrupted ultrasonic disruptor UD-200 at 60 W for 4 min intermittently in an ice bath. Cell extract was obtained by centrifugation at 12000 rpm and 4°C for 10 min. The cell extract was subjected to aminoacylase assay at different set of parameters.

Aminoacylase Activity Assay

This asssay was adapted with modification from Story and co-workers[4]. Aminoacylase activity was determined in 0.5 mL assay mixtures containing 0.2 mL of aminoacylase enzyme in 50 mM Tris-HCl (pH7.5) and 0.3 mL of 10 mM of N-acetyl-$_L$-methionine (NAMET) substrate. The assay mixtures was incubated in waterbath shaker 200 rpm for 30 min. The assay temperature were set at 30, 40, 50, 60, 70, 80 and 90 °C to determine the optimum temperature. The reaction was stopped using 0.5 mL of 10% Trichloroacetic acid (TCA). The precipitated protein was removed by centrifugation. The 1 mL supernantant was mixed with 0.25 mL of 3% ninhydrin solution and 0.25 mL of 250 mM acetic-cyanide (pH5.0) buffer. The mixture was boiled for 15 min and cooled to ambient temperature by the addition of 1.5 mL of 50% (v/v) isopropanol. The liberated L-methionine was measured using at absorbance wavelenghth of 570 nm (A_{570nm}). One unit of aminoacylase activity was defined as the amount of the enzyme required to produce 1 µmole of L-methionine in 30 min at 60°C at pH 7.5.

Identification of the Thermophilic Bacterium

Identification of bacteria was carried out using analysis of 16S rRNA gene sequence through genomic DNA extraction, PCR amplification using primers 27F and 1492R (Table 1), gel electrophoresis, gel purification, cloning into pJET/1.2 blunt end vector (Fermentas, Canada), transformed into *Escherichia coli* TOP 10 (Invitrogen, USA), sequencing of 16S rRNA and identified using Basic Local Alignment Search Tool (BLAST) [5]. Evolutionary traits of the identified bacterium was analyzed using MEGA 7.0 software [11].

Table 1: Primers for PCR amplification used throughout the study

Primers	Sequence (5'→3')
27F	AGAGTTTGATCTGGCTCAG
1492R	CGGTTACCTTGTTACGACTT
AMAF	ATGACAAAGGAAGAAATCAAACGG
AMAR	TCAATCGTAAAGCGCGGGT

Molecular Investigation of Gene Encoding Aminoacylase Gene *(ama)*

Amplification of *ama* gene encoding aminoacylase from *Geobacillus* sp. strain SZN was carried out using primers AMAF and AMAR (Table 1) according to standard PCR procedure. Analysis of the aminoacylase nucleotide sequence and database similarity was performed by using Basic Local Alignment Search Tool [5] and Biology Workbench [6]. On the other hand, SOPMA software [12] was used for secondary structure analysis, utilizing aminoacylase amino acids sequence.

Results

Isolation and Screening of Aminoacylase Producer

Round and yellowish colonies (data not shown) were detected on LB agar plate following incubation at 60 °C. Further incubation of a loopful bacteria colony into LB broth had shown bell shaped graph (Figure 1A), indicated that the bacteria exponetially growth from 30 to 60°C and started to deminish after 70°C and above.

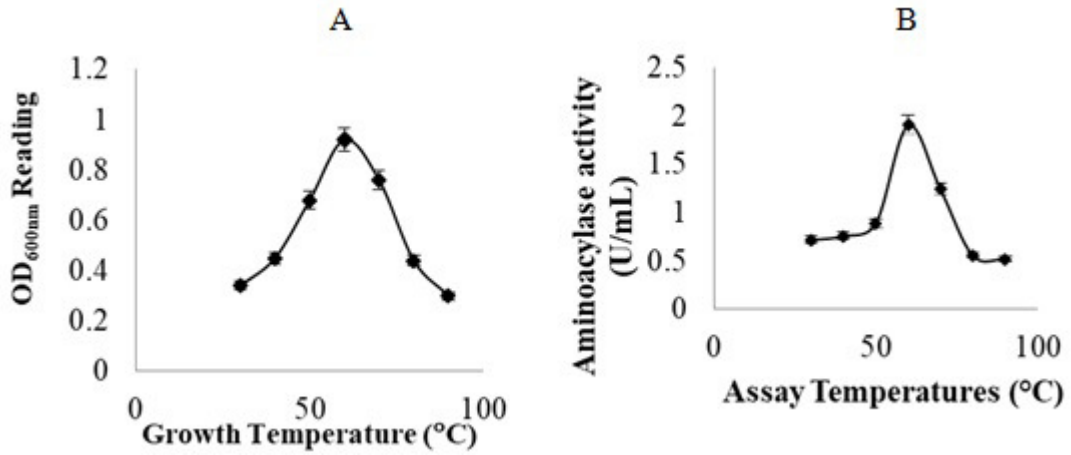

Figure 1. Screening of aminoacylase producer. (A) The OD_{600nm} reading for optimization of bacteria growth temperatures. (B) Determination of aminoacylase optimum temperature.

A common method to estimate the concentration of bacteria or other cells in a liquid by using OD_{600} [8]. Measuring the concentration can indicate the phase of cultured cell growth, whether it is in lag phase, exponential phase, or stationary phase. OD_{600} is preferable to UV spectroscopy when measuring the growth over time of a cell population because at this wavelength, the cells will not be killed as they would when under too much UV light. The reading of OD_{600} shows that the bacteria growth culture turbidity was gradually increased from temperatures 30, 40 and50 °C with OD_{600} reading of 0.34, 0.45 and 0.68, respectively**.** The optimum gowth occured at temperature 60°C with OD_{600nm}reading of 0.92 and gradually decreased to 0.76 ,0.44 and 0.30 at respective temperatures of 70, 80 and 90°C.

Once optimum temperature for bacteria growth was determined at 60 °C, isolates were further analysed for screening of aminoacylase activity at different assay temperatures ranging from 30 to 90°C. Result shows that highest aminoacylase activity occurred at temperature 60 °C with 1.91 U/mL activity and the activity started to decrease when temperature were increased to 70, 80 and 90°C with respective enzyme activity at 1.25, 0.56 and 0.52 U/mL (Figure 1B). This result is in agreement with bacteria optimum growth temperature which is 60°C that we believed the protein produced by organisms will favor their catalytic activity close to their native conditions.

Molecular Identification of the Isolated Bacterium

The 1513 nucleotides deduced from the positive recombinant were analyzed using Basic Local Alignment Search Tool (BLAST). The result demonstrated that the isolate was fall within the genus *Geobacillus* (data not shown). The approximate 1.5kb of 16S gene sequence matches 99% of the complete 16S gene sequence from variety species of *Geobacillus* with different strains at NCBI Genbank. Thus, the newly isolated bacteria were assigned as *Geobacillus*strain SZN. Based on 16S rRNA sequence alignment, phylogenetic tree was constructed (Figure 2) for the isolated strain. The evolutionary history was inferred using the Neighbor-Joining method [13]. Constructed phylogenetic tree revealed that closest homolog of *Geobacillus*strain SZN was *Geobacillus*sp. 70PC53 (EU860293.1).

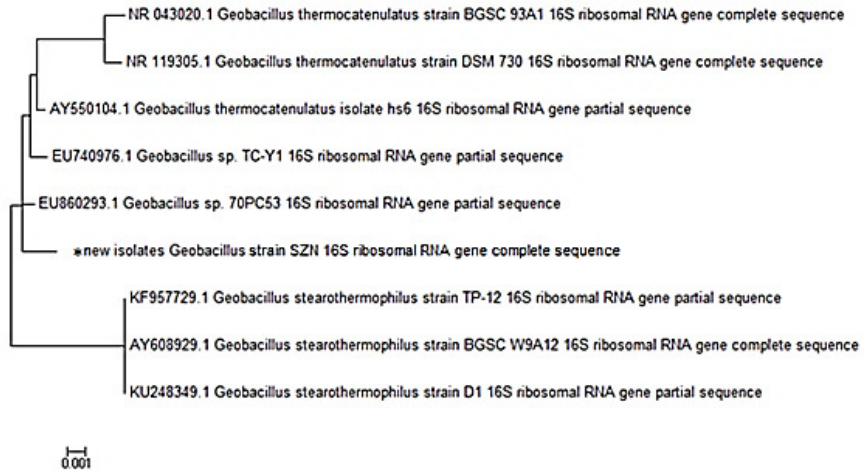

Figure 2. Phylogenetic tree of 16S gene from *Geobacillus* strain SZN.

Molecular Investigation of Gene Encoding Thermophilic Aminoacylase

Full sequence of 1113 bp of nucleotide of aminoacylase was successfully excised from *Geobacillus*strain SZN. Complete open reading frame (ORF) nucleotide sequence of *ama* gene and its deduced primary structure of the protein from *Geobacillus* strain SZN are shown in Figure 3. The sequence analysis will be helpful information for wild-type purification step by providing information about the characteristics of the enzyme. The ORF containing 1113 bp, start with ATG start codon and end with TGA stop codon. The gene consisted of 370 amino acid residues with calculated molecular mass of approximetly 42 kDa by using Protparam tools which similar to Sakayan et al.[14].

```
1    atgacaaaggaagaaatcaaacgactcgtcgatgaagtgaaaacggacgtcatcgcctgg
       M  T  K  E  E  I  K  R  L  V  D  E  V  K  T  D  V  I  A  W  20
61   cgccgtcatttgcatgcccatccggaattgtcgttccaagaagagaaaacagcgcagttt
       R  R  H  L  H  A  H  P  E  L  S  F  Q  E  E  K  T  A  Q  F  40
121  gtctatgagacgctgcaatcattcggtcatcttgaactttcgcggccgacgaaaacgagc
       V  Y  E  T  L  Q  S  F  G  H  L  E  L  S  R  P  T  K  T  S  60
181  gtcatggcgcggctcattggccaacagccaggccgggtcgtcgccattcgcgctgatatg
       V  M  A  R  L  I  G  Q  Q  P  G  R  V  V  A  I  R  A  D  M  80
241  gacgcattgccgattcaagaggaaaacacgtttgagtttgcctcaaaaaacccaggcgtg
       D  A  L  P  I  Q  E  E  N  T  F  E  F  A  S  K  N  P  G  V 100
301  atgcatgcgtgcggacatgacggccatacggcgatgcttctcgggacggcgaaaatttc
       M  H  A  C  G  H  D  G  H  T  A  M  L  L  G  T  A  K  I  F 120
361  tcccagctgcgcgatgacattcgcggtgaaatccgctttttgttccaacacgcggaagaa
       S  Q  L  R  D  D  I  R  G  E  I  R  F  L  F  Q  H  A  E  E 140
```

```
421  ttgttccccggcggggcggaggagatggtgcaagctggtgtcatggacggggtggacgtc
       L  F  P  G  G  A  E  E  M  V  Q  A  G  V  M  D  G  V  D  V 160
481  gtcatcggcactcacctttggtcgccgctcgagcgcggaaaaatcggcattgtgtatggg
       V  I  G  T  H  L  W  S  P  L  E  R  G  K  I  G  I  V  Y  G 180
541  ccgatgatggccgcacccgaccgctttttcatccgcatcatcggcaaaggcggccacggg
       P  M  M  A  A  P  D  R  F  F  I  R  I  I  G  K  G  G  H  G 200
601  gcgatgccgcaccaaacgatcgatgcgatcgccatcggagcgcaagtcgtgacgaacttg
       A  M  P  H  Q  T  I  D  A  I  A  I  G  A  Q  V  V  T  N  L 220
661  cagcacattgtctcgcgctatgtcgacccgctcgagccgcttgttctgtccgtgacgcaa
       Q  H  I  V  S  R  Y  V  D  P  L  E  P  L  V  L  S  V  T  Q 240
721  tttgtggcgggtacggcgcataatgtcctgcctggggaggtcgaaatccaagggacagtg
       F  V  A  G  T  A  H  N  V  L  P  G  E  V  E  I  Q  G  T  V 260
781  cgcacgttcgatgagacgctgcggcgcacggtgccgcaatggatggagcgcattgtcaaa
       R  T  F  D  E  T  L  R  R  T  V  P  Q  W  M  E  R  I  V  K 280
841  gggatcaccgaagcgcacggcgcctcgtatgagtttcgatttgactacggctaccgcccg
       G  I  T  E  A  H  G  A  S  Y  E  F  R  F  D  Y  G  Y  R  P 300
901  gtcatcaactacgatgaaggtgacccgcgtcatggaggaaacggcgtgcgagctgttcgg
       V  I  N  Y  D  E  G  D  P  R  H  G  G  N  G  V  R  A  V  R 320
960  cgaagaggcagtggtccgcttgaaaccgaacatgggcggcgaagatttctccgcctttt
       R  R  G  S  G  P  L  E  T  E  H  G  R  RR  F  L  R  L  F 340
1021 gcaaaaagcgcccggcagctttttctacgtcggcgcgggcaatgtagaaaaaggcatcgt
       A  K  S  A  R  Q  L  F  L  R  RR  G  Q  C  R  K  R  H  R 360
1081 ttacccgcaccaccacccgcgctttacgattga
       L  P  A  P  PP  A  L  Y  D  -                    370
```

Figure 3. The ORF sequence of*ama* gene from *Geobacillus*strain SZN. Numbers of nucleotide and amino acid are indicated at extreme left and right sides, respectively.

The ORF sequence of *ama* was analyzed using Basic Local Allignment Tool (BLAST) tools. Result shows that *Geobacillus* strain SZNhad 99% identity with *ama* orf4 gene (Y08751) and N-carbamyl-L-amino acid amidohydrolase (Y74289) from *B. stearothermophilus* (data not shown). Multiple sequences alignment of aminoacylase strain SZN with *Bacillus stearothermophilus* (X74289), *Geabacilluskaustophilus* (Q5KUV0), *Pyrococcushorikoshii* (O58453), *Pyrococcusabsyii* (Q9V2D3), *Bacillus subtilis* (1YSJ), and *Staphylococcus aereus*(4EWT) with amino acid sequence similarities 100, 97, 44, 46, 45, and 39%, respectively. From the construction of the phylogenetic tree, aminoacylase SZN positioned itself closely to aminoacylase from*B. stearothermophilus* (Figure 4).

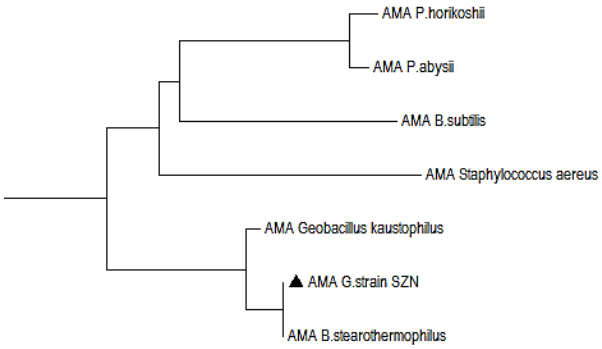

Figure 4. Phylogenetic tree of aminoacylase strain SZN gene.

Aminoacylase enzyme is classified into M20 family of metallopetidases, in which zinc is needed as an essential metal for catalytic activity [15]. The alignments clearly demonstrate that these aminoacylase sequences share highly conserved regions across different species (Figure 5). Previous reported findings from Tanimoto and colleagues [3] had revealed the positions of metal binding site residues for *Pyrococcushorikoshii*was found at His106, Glu139, Glu140 and His164 whilecatalytic residues at H198 and A260.

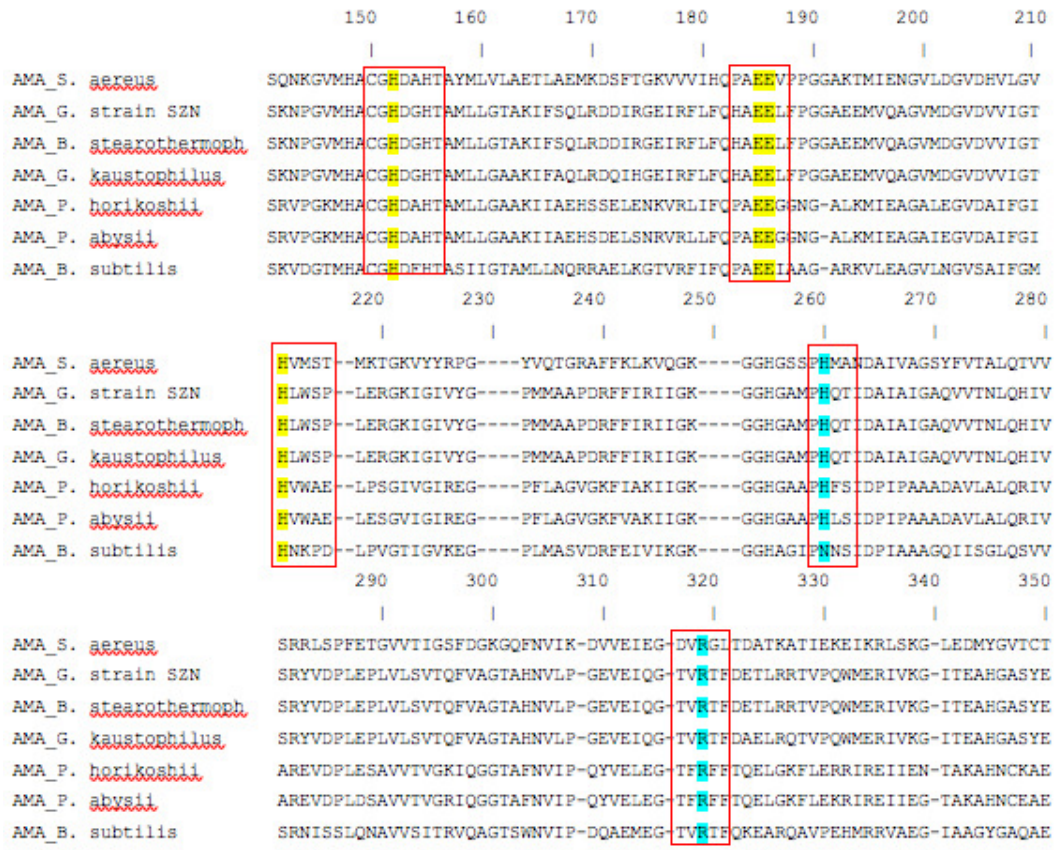

Figure 5. Multiple sequences alignment of aminoacylase strain SZN with *Bacillus stearothermophilus (*X74289), *Geabacilluskaustophilus* (Q5KUV0), *Pyrococcushorikoshii*

(O58453), *Pyrococcusabsyii* (Q9V2D3), *Bacillus subtilis* (1YSJ), and *Staphylococcus aereus* (4EWT). The conserved region among the sequence was marked with red box. The highlighted amino acid residuesin yellow and turqoise represents predicted metal binding site and active site residues, respectively, for aminoacylasew from*Geobacillus*sp. strain SZN.

Since allignment results found that, the predicted sites for metal binding sites and active sites from *Pyrococcushorikoshii*was conserved in most of species including aminoacylase SZN, thus, metal binding site for aminoacylase SZN was predicted at His106, Glu139, Glu140, and His165 and active sites was predicted at His199 and Arg261. Secondary structure prediction of aminoacylase SZN has clearly demonstrated that two of the residues for metal binding sites, Glu139 and Glu140, are located in α-helix. Nevertheless, His106 and His165 are estimated in random coil (Figure 6). The Arg261 as the catalytic residue is estimated at α-helix while His199 residue is estimated in β-turn.

Figure 6. Aminoacylase strain SZN secondary structure estimation. All the predicted residues for metal binding sites and catalytic sites were marked with red and black underline, respectively. The respective alphabets consist of c, h, t, and e represents respective secondary structure of random coil, α-helix, β-turn and extended strand. The secondary structure estimation for catalytic sites was underlined with black.

DISCUSSION

Isolation and identification of aminoacylase producing bacteria

A new *Geobacillus* strain was assigned as *Geobacillus*sp. strain SZN. Since the bacteria growth and aminoacylase activity was detected to be optimum at 60°C, *Geobacillus*sp. strain SZN can be grouped as thermophile. *Bacillus* and the related genera are one of the most interested groups of bacteria in industrial biotechnology by taking advantage of their enzymes which mostly showed resistance to high pHs and temperatures especially in harsh industrial and many pharmaceutical and agricultural processes [16]. The ability of different species to ferment in acidic to alkaline pH ranges, presence of many thermophiles in the genus has led

to the development of a variety of new commercial enzyme products with the desired temperature, pH activity, and stability properties to address variety specific applications [17].

Molecular Investigation of Aminoacylase SZN Sequence

It is important to know the properties and mechanism especially the structural adaptations at high temperatures so that further studies for better stabilities of aminoacylase enzyme can be more manipulated. In this report, aminoacylase SZN shares the similar catalytic residues between various species reported before. Several parts of conserved residues were found among the species when the amino acid sequence was aligned, even though the similarity was below 50%. The predicted metal binding (Glu139 and Glu140) and catalytic sites (Arg261) which formed in α-helix structure, had inferred importance of α-helix secondary structure with enzyme thermostability and activity stability. High portion of α-helix structure at the active sites assists the stability structure of the aminoacylase at high temperatures because α-helix contains strong hydrogen bonds derived from bonding of carbonyl group of amino acid with amino group of other amino acids that restores the secondary structure of a protein. In addition, the GC content also played a necessary role in adaptation temperatures and it was hypothesized that the thermostability was given to the genetic materials in high GC organisms [18], the percentage of GC content from this aminoacylase SZN is 55%, and this high amount of hydrogen bonds can assist the thermostability of the aminoacylase SZN.This reported data was helpful information for the next researcher who wants to emphasize in depth studies about the importance of α-helix structure in thermostability mechanisms of thermostable proteins particularly in thermostable aminoacylase.

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