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Research Article
Characterization of the Type 2 L-Asparaginase Gene in Thermohalophilic Bacterial from Wawolesea Hot Springs, Southeast Sulawesi, Indonesia

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Abstract

Background and Objective: Type 2 L-asparaginase enzyme can be used as a cancer therapy agent and prevent acrylamide formation in food products. Enzymes produced by thermohalophilic bacteria can provide high activity at high temperatures so they are needed on an industrial scale. Hence, this study aims to determine the characteristics of the gene encoding type 2 L-asparaginase enzyme in the thermohalophilic bacterial isolate CAT3.4. **Materials and Methods:** This research is a type of exploratory research. The characteristics of the gene encoding type 2 L-asparaginase were determined using the PCR technique using the primer pairs AsnBac2-F2 (5'-CTCACGGGAATCTCCATAACTC-3') and AsnBac2-R2 (5'CAGCGATGTAACAGACAGCATC-3'). The characterization process was carried out in stages: Isolation of genomic DNA using a modified alkali-lysis method, nucleotide and protein similarity analysis using BLASTn analysis on the NCBI website, construction of a phylogenetic tree using the MEGAX program, restriction enzyme mapping and amino acid analysis using the Bioedit program. **Results:** The characterization results showed that the PCR product has a size of 1594 bp with a CDS of 1128 bp, has a similarity value of 100% with *Bacillus subtilis*, has seven restriction enzymes as molecular markers for the type 2 L-asparaginase gene at the species level: *Bsr*GI, *Dra*I, *Eco*RV, *Hind*III, *Hpy*CH4IV , *Ssp*I and *Taq*I, have dominant hydrophilic regions and are in the same subclass as *Bacillus subtilis* strain GOT9. **Conclusion:** The target gene was similar to the gene encoding type 2 L-asparaginase from *Bacillus subtilis* with a max identity of 98.85%, query coverage value of 100% and E-value of 0.

Key words: Type 2 L-asparaginase gene, thermohalophilic bacteria, *Bacillus subtilis*, Wawolesea hot springs

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The L-asparaginase enzyme is a type of hydrolase enzyme that catalyzes the hydrolysis reaction of asparagine into aspartic acid and ammonia[1]. The L-asparaginase is widely developed in various fields, such as the biomedical and food industries. The L-asparaginase was developed in the medical world to treat leukemia[2]. The L-asparaginase can prevent acrylamide formation in food[3].

The L-asparaginase is present in various organisms, including animals, plants and bacteria, but is absent in humans[4]. However, L-asparaginase produced from this organism still needs to improve, including low activity and stability at high temperatures, making the enzyme less attractive for application on an industrial scale. Using L-asparaginase on an industrial scale prioritizes L-asparaginase, which has optimum activity and stability at high temperatures because it is more efficient in accelerating the L-asparagine hydrolysis reaction. The L-asparaginase, which has optimum activity and stability at high temperatures, can be explored in microorganisms living in extreme environments, such as environments with high temperatures and salinity. Bacteria were chosen as a profitable source of L-asparaginase because bacteria can produce more enzymes, the price is economical and enzyme production is easy to regulate according to desired characteristics[3,5]. One type of bacteria that is a source of L-asparaginase with high capability and stability to temperature is thermohalophilic bacteria.

Research conducted by Muzuni *et al*.[6] succeeded in characterizing thermohalophilic bacteria from the Wawolesea hot springs, which have L-asparaginase enzyme activity. Then, Muzuni *et al*.[6] identified an isolate of the thermohalophilic CAT3.4 bacteria from the Wawolesea hot springs, which is phonetically similar to *Bacillus subtilis* subsp., subtilis BS-2.

The ability of the thermohalophilic CAT3.4 bacterial isolate to produce L-asparaginase needs further development so that L-asparaginase production in Indonesia can be increased significantly. Currently, the production of L-asparaginase from the thermohalophilic bacterial isolate CAT3.4 still needs to be improved because of the need for more information on the sequence data of the L-asparaginase gene. Gene characterization is important because it can provide information regarding L-asparaginase gene sequence data.

The L-asparaginase in bacterial cells is divided into type 1 L-asparaginase (*Ans*A) and type 2 L-asparaginase (*Ans*Z). Type 1 L-asparaginase is expressed constitutively in the cytoplasm. Type 2 L-asparaginase exists in anaerobic conditions in the periplasmic space and has a higher specificity for hydrolyzing asparagine[7]. Type 2 L-asparaginase also has a higher affinity for L-asparagine than type 1 L-asparaginase[8,9]. The cause of the higher affinity and specificity of the type 2 L-asparaginase enzyme for L-asparagine compared to type 1 L-asparaginase requires in-depth study. The primary study that needs to be carried out to determine the nature of the enzyme is to characterize the enzyme and its coding gene. An essential initial step to take is the characterization of the gene encoding type 2 L-asparaginase, which includes a similarity index analyzed using the BLAST program, a phylogenetic tree to describe the closeness of each bacterium that has the type 2 L-asparaginase gene, the restriction enzyme cutting site and its hydrophobicity profile. Therefore, research on the characterization of the *Ans*Z gene, encoding enzyme type 2 L-asparaginase, thermohalophilic CAT3.4 bacteria from Wawolesea Hot Springs, Southeast Sulawesi, Indonesia, must be carried out.

MATERIALS AND METHODS

This research was conducted from June to December, 2022 at the Microbiology Laboratory and Biomolecular Laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, Halu Oleo University, Southeast Sulawesi, Indonesia.

Making NA media (nutrient agar): The NA media is used as a bacterial rejuvenation media. The composition of NA media is agar, beef extract, yeast extract, NaCl and peptone. The NA media was made by mixing eight grams of NB (Nutrient Broth) and 20 g of agar in 1000 mL of sterile distilled water. The media solution is then heated and homogenized with a LED Magnetic Hotplate Stirrer Type MS7-H550-S. Then, the media was sterilized using an autoclave Type LDZX-100B with a pressure of 1 atm 121°C for 15 min.

NB media (nutrient broth): The NB media (nutrient broth) is used as a bacterial growth medium. The composition of NB media is 0.03 g of beef extract, 0.3 g of yeast extract, 0.1 g of NaCl, 0.1 g of peptone and dissolved in 20 mL of sterile distilled water. The NB media solution was then homogenized. Next, the media was sterilized using an autoclave Type LDZX-100B with a 1 atm pressure and 121°C for 15 min.

Sterilization of tools and media: Sterilization of tools and media uses an autoclave using the pressurized hot steam method at a temperature of 121°C for 15 min[10].

Table 1: Composition of the type 2 L-asparaginase gene amplification mix solution

Solution	Final concentration
Genomic DNA of bakteri termohalofilik CAT3.4	10.0 ng
AsnBac2-F2 Primer	0.5 mM
AsnBac2-R2Primer	0.5 mM
2x master mix	1.0x
dH_2O	up to 10.0 µL
Total volume	10 µL

Rejuvenation of CAT3.4 thermohalophilic bacterial isolates: Rejuvenation of CAT3.4 thermohalophilic bacterial isolates is carried out by inoculating the bacterial isolates into slanted NA (nutrient agar) media using the scratch method using a round loop. Then, it was incubated in an incubator at 50°C for 24 hrs[11].

Genomic DNA isolation of CAT3.4 thermohalophilic bacterial isolates: Genomic DNA isolation of CAT3.4 thermohalophilic bacterial isolates was carried out using a modified alkaline lysis method[11]. Bacteria were cultured in NB media for 24-48 hrs at 50°C. A total of 1.5 mL of bacterial culture was put into a microcentrifuge tube and centrifuged at 10,000 rpm for 10 min at a temperature of 27°C. The pellet was taken and 100 µL of solution I (50 mM glucose, 25 mM Tris-Cl, 10 mM EDTA) was added to lyse the bacterial cell wall and vortexed for 5 min. Then 200 µL of solution II (0.2 N NaOH, 1% SDS) was added to the sample to lyse the bacterial cell walls and vortexed. Then, the sample was incubated in a water bath for 30 min and vortexed every 10 min. Finally, 300 µL of solution III was added to the sample (60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid, 28.5 mL H_2O), then vortexed and centrifuged at a speed of 10,000 rpm for 10 min at a temperature of 4°C.

The supernatant was taken and 1x the phenol-chloroform (PC) volume was added and vortexed until homogeneous. Next, the sample was centrifuged at 10,000 rpm, 4°C for 10 min. The supernatant was taken and added with 0.1 volume of 3 M sodium acetate pH 5.2, added with 2x the volume of absolute ethanol and then incubated at -20°C for 24 hrs. Next, the sample was centrifuged at 12,000 rpm, temperature 4°C, for 20 min. After that, the DNA pellet was washed with 500 µL of 70% alcohol. Then dried and dissolved in 20-50 µL sterile dH_2O.

Primer design: Primer design was carried out by collecting type 2 L-asparaginase gene sequences from various species of *Bacillus subtilis* bacteria obtained from GenBank data[11]. The gene sequence is aligned using the ClustalW Alignment Bioedit program to determine forward and reverse based on the most conserved sequence. The sequences that were used as primers were AsnBac2-F2 primer (5'-CTCACGGGAATCTTCCCATAACTC-3') and AsnBac2-R2 primer (5'-CAGCGATGTAACAGACAGCATC-3').

Type 2 L-asparaginase gene amplification by PCR technique: The L-asparaginase gene of thermohalophilic bacteria CAT3.4 was amplified with the composition as shown in Table 1. The PCR process consists of initial denaturation at 94°C for 5 min. The PCR process consisted of 35 cycles, which included denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 90 sec and final extension at 72°C for 5 min. The PCR product measuring approximately 1600 bp was confirmed by electrophoresis.

Sequencing and sequence characterization: Purification and sequencing of PCR products was carried out at 1st Base Singapore. Sequencing results were characterized using MEGA 11 Version 11.0.13, which is used for the analysis of phylogenetic tree construction based on nucleotide sequence and amino acid sequence, ClustalW Alignment Bioedit program for analysis of restriction enzyme cutting sites, hydrophobicity and sequence alignment analysis of type 2 L-asparaginase encoding gene isolates of thermohalophilic bacteria CAT3.4 with L-asparaginase encoding gene sequences in GenBank, and the Expasy program was used for amino acid sequence analysis.

RESULTS AND DISCUSSION

Quality of genomic DNA isolated from thermohalophilic bacteria CAT3.4: The results of isolated genomic DNA from isolated thermohalophilic bacteria CAT3.4 were visualized by electrophoresis to see the quality of the genomic DNA. Electrophoresis is the standard method used to determine the quality and quantity of isolated DNA[12]. Electrophoresis moves DNA using the electric field generated from electrodes to separate charged compounds[11]. Visualization of genomic DNA isolates CAT3.4 thermohalophilic bacteria shown in Fig. 1.

The electrophoresis results showed that the genomic DNA of the thermohalophilic bacteria CAT3.4 was successfully isolated (Fig. 1). Even though the electrophoresis results still showed a smear pattern indicating the presence of slightly degraded DNA, the DNA could already be used as a template in PCR. According to Matmarurat *et al.*[12], the smear pattern from the electrophoresis results indicated contamination from proteins, RNA and DNA, which were degraded due to excessive physical movement during the DNA isolation. Good DNA quality is characterized by not fragmented DNA and the bands appear thick and clear without a smear pattern[13].

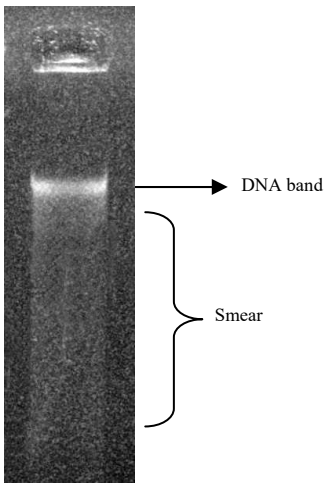

Fig. 1: Results of genomic DNA electrophoresis of isolates of thermohalophilic bacteria CAT3.4 on 1% agarose gel

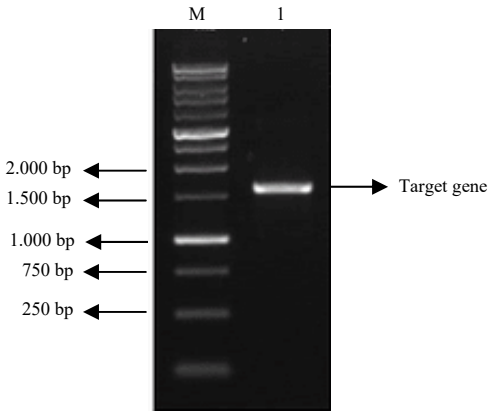

Fig. 2: Results of electrophoresis of PCR gene L-asparaginase type 2 product isolates of thermohalophilic bacteria CAT3.4 using AsnBac2-F2 and AsnBac2-R2 primers on agarose gel
M: Marker 1 kb ladder and 1: PCR product

Amplification of type 2 L-asparaginase gene isolates CAT3.4 thermohalophilic bacteria: Amplification of the gene encoding the enzyme type 2 L-asparaginase isolates of thermohalophilic bacteria CAT3.4 was successfully carried out using PCR technique. Polymerase Chain Reaction (PCR) is a fast procedure for amplifying specific DNA sequences *in vitro* by using two primers hybridized to opposite strands so that they flank the target DNA[14].

Electrophoresis results showed that the type 2 L-asparaginase gene amplified with primers AsnBac2-F2 and AsnBac2-R2 produced a PCR product measuring around 1500 bp (Fig. 2). The amplification success was indicated by forming a single band on the electrophoresis gel parallel to the 1500 bp band from the 1 kb ladder marker.

Sequencing of the type 2 L-asparaginase gene: Gene sequence sequencing aims to produce complete sequence data from the sequencing results. The sequencing process uses forward and reverse primers separately, which will obtain data in the form of peak electropherograms of complementary Single-Stranded DNA (ssDNA) from forward or reverse primers. If one of the ssDNA strands from the forward or reverse has the wrong nucleotide, it can be replaced using data from the complementary strand. Heyl and Backofen[15] explained that good sequencing data is indicated by peaks with high peaks separated from each other.

The DNA sequence measuring 1594 bp (Fig. 3) was amplified using primer AsnBac2-F2 and primer AsnBac2-R2. The AsnBac2-F2 primer was designed at 239 bp before the start codon and the AsnBac2-R2 primer was designed at 227 bp after the stop codon. Both primers have a GC content of 48%, measuring 21 bp. According to Kumar and Kaur[16], good quality primers have a GC content of 40-60% with a length ranging from 18-30 bases, without dimers and hairpins.

These results show that the coding sequence (CDS) of the type 2 L-asparaginase gene measures 1128 bp from the start codon (240th nucleotide, ATG) to the stop codon (1367th nucleotide, TGA). Jia *et al.*[4] reported that, the type 2 L-asparaginase gene was successfully amplified from *Bacillus subtilis* B11-06 genomic DNA measuring 1128 bp.

Similarity index analysis: Similarity index analysis was carried out using the BLAST found on the NCBI website. The BLAST (Basic Local Alignment Sequence Tools) analysis compared the type 2 L-asparaginase gene sequences of isolates of thermohalophilic bacteria CAT3.4 with sequences from other bacteria. In addition, BLAST analysis shows changes in nucleotides (mutations) from existing samples with gene sequences in GenBank. Yusnaini *et al.*[17] reported that, the alignment results carried out with the BLAST program from the NCBI website could indicate changes in the nucleotide sequence (mutations) in the enzyme coding gene fragment. The results of the BLAST analysis were presented in Fig. 4.

Based on BLASTn analysis, the CAT3.4 thermohalophilic bacterial isolate has a very high similarity to the *Bacillus subtilis* group of bacteria with a Max identity of 98.85%, Query coverage values up to 100% and all E-values of the sequences are 0 (Fig. 4). This means that the type 2 L-asparaginase gene

```
   1  CTCACGGGAATCTCCATAACTCATAACATTCCCACCTTACTGAATTGCAATCAAAATATA   60
  61  GTGACTGGTCTATTATCTTGATTCAATCATCAATTGTCAAGAAAAATTCATTGTATGAAA  120
 121  AGACAAAAAAAGAAGGATATGACAACAAAAAATACTGAGAGAAAAGCTGACTGATCTTTT  180
 181  GACTGAATAGATAAAATACTCAATGATTAATCATCATATGGATGTAAGGAGAGAAATAGA  240
 241  TGAAAAAACAACGAATGCTCGTACTTTTTACCGCACTATTGTTTGTTTTTACCGGATGTT  300
 301  CACATTCTCCTGAAACAAAAGAATCCCCGAAAGAAAAAACGCAGACACAAAAAGTCTCTT  360
 361  CGGCTTCTGCCTCTGAAAAAAAGGATCTGCCAAACATTAGAATTTTAGCGACAGGAGGCA  420
 421  CGATAGCTGGTGCCGATCAATCGAAAACCTCAACGACTGAATATAAAGCAGGTGTTGTCG  480
 481  GCGTTGAATCACTGATCGAGGCAGTTCCAGAAATGAAGGACATTGCAAACGTCGGCGGCG  540
 541  AGCAGATTGTTAACGTCGGCAGCACAAATATTGATAATAAAATATTGCTGAAGCTGGCGA  600
 601  AACGCATCAACGACTTGCTCGCTTCAGATGATGTAGACGGAATCGTCGTGACTCATGGAA  660
 661  CAGATACATTGGAGGAAACCGCTTATTTTTTGAATCTTACCGTGAAAAGTGATAAACCGG  720
 721  TTGTTATTGTCGGTTCGATGAGACCTTCCACAGCCATCAGCGCTGATGGGCCTTCTAACC  780
 781  TGTACAATGCAGTGAAAGTGGCAGGTGCCCCTGAGGCAAAAGGGAAAGGGACGCTTGTTG  840
 841  TTCTTAACGACCGGATTGCCTCAGCCCGATATGTCACCAAAACAAACACAACCACAACAG  900
 901  ATACATTTAAATCAGAAGAAATGGGCTTCGTCGGAACAATTGCAGATGATATCTATTTTA  960
 961  ATAATGAGATTACTCGTAAGCATACGAAGGACACGGATTTCTCGGTTTCTAATCTTGATG 1020
1021  AGCTGCCGCAGGTTGACATTATCTATGGATACCAAAATGACGGAAGCTATCTGTTTGACG 1080
1081  CTGCTGTAAAAGCCGGAGCAAAGGGGATTGTATTTGCCGGTTCTGGGAACGGGTCTTTAT 1140
1141  CTGACGCAGCCGAAAAAGGGGCGGACAGCGCAGTCAAAAAAGGCGTCACAGTGGTGCGCT 1200
1201  CTACCCGCACGGGAAATGGTGTCGTCACACCAAACCAAGACTATGCGGAAAAGGACCTGC 1260
1261  TGGCATCGAACTCTTTAAACCCCCAAAAAGCACGGATGTTGCTGATGCTTGCGCTTACCA 1320
1321  AAACAAATGATCCTCAAAAAATCCAAGCTTATTTCAATGAGTATTGAAGAAAAGAAGGCG 1380
1381  AATAAGCCTTCTTTTTTTGGCTTTTTAGGACCAATAATGACCTATGAATCTTAAAATTTC 1440
1441  TTTAAAAATAAGCCAAAATTACCCTTTACTTAATTAATTTGGTAACGTAATATATTTGGA 1500
1501  GAATTTGTTACAAAAAAAGGAGGATATTATGAAATTTGTAAAAAGAAGGATCATTGCACT 1560
1561  TGTAACAATTTTGATGCTGTCTGTTACATCGCTG                            1594
```

Fig. 3: Amplified gene sequences
Underline: primer sequence of AsnBac2-F2 and AsnBac2-R2, bold: Start (ATG) and Stop (TGA) codons

	Description	Common Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Bacillus subtilis strain GOT9 chromosome, complete genome	Bacillus subtilis	2034	2034	100%	0.0	98.85%	4119195	CP047325.1
☑	Bacillus subtilis strain BS16045, complete genome	Bacillus subtilis	2034	2034	100%	0.0	98.85%	4165121	CP017112.1
☑	Bacillus subtilis strain TO-A JPC, complete genome	Bacillus subtilis	2034	2034	100%	0.0	98.85%	4090708	CP011882.1
☑	Bacillus subtilis TOA, complete genome	Bacillus subti...	2034	2034	100%	0.0	98.85%	4057487	CP005997.1
☑	Bacillus subtilis strain JCL16 chromosome, complete genome	Bacillus subtilis	2006	2006	100%	0.0	98.41%	4101682	CP054177.1
☑	Bacillus subtilis strain SG6, complete genome	Bacillus subtilis	2006	2006	100%	0.0	98.41%	4079669	CP009796.1
☑	Bacillus subtilis strain MB9_B6 chromosome, complete genome	Bacillus subtilis	2001	2001	100%	0.0	98.32%	4087720	CP045818.1
☑	Bacillus subtilis strain MB9_B4 chromosome, complete genome	Bacillus subtilis	2001	2001	100%	0.0	98.32%	4105407	CP045819.1
☑	Bacillus subtilis strain MB9_B1 chromosome, complete genome	Bacillus subtilis	2001	2001	100%	0.0	98.32%	4263919	CP045820.1

Fig. 4: Results of BLAST analysis: Nucleotide sequence alignment of the L-asparaginase type 2 encoding gene between thermohalophilic bacterial isolates CAT3.4 and bacteria present in GenBank

sequence from the thermohalophilic bacterium CAT3.4 has similarities to the *Bacillus subtilis* group of bacteria. Kilinc *at al.*[18] explains that the score indicates the accuracy of the sequence alignment value. The higher the score obtained, the higher the homology level of the two sequences. The E-value is an estimated value that provides a statistically significant measure of both sequences. The E-value is 0 (zero), indicating that the two sequences are identical.

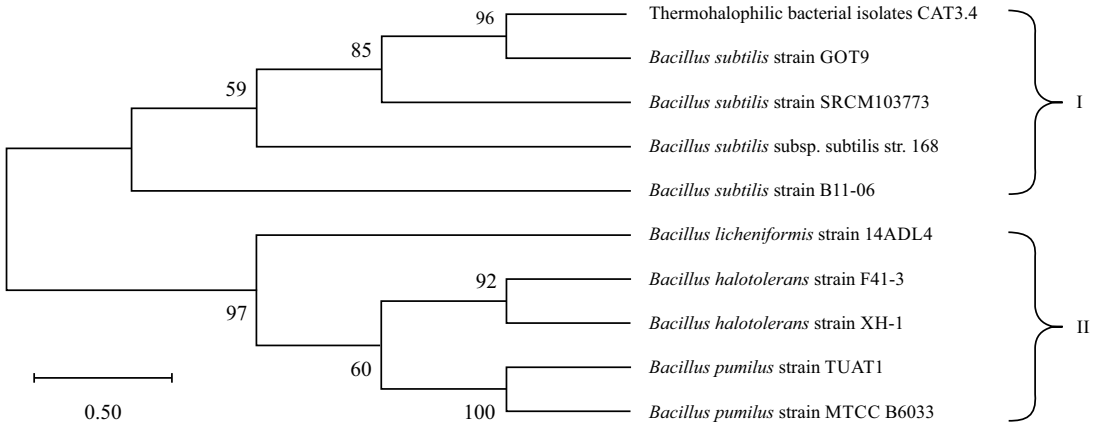

Fig. 5: Phylogenetic tree constructed using the maximum likelihood algorithm which shows the relationship between the thermohalophilic bacterial isolate CAT3.4 and the reference strain for members of the genus *Bacillus* based on the nucleotides of the type 2 L-asparaginase gene

Numbers on the branches indicate bootstrap values with 1000x replication. The scale indicates the substitution/mutation value that occurs from 5 per 100 nucleotide sequences of the L-asparaginase type 2 gene

Phylogenetic tree analysis using the MEGA X program: Phylogenetic tree analysis is fundamental to determining the kinship among organisms arranged based on similarities in genes, nucleotides and amino acids. Phylogenetic tree analysis in this study was carried out based on the nucleotide sequence of the L-asparaginase gene type 2 isolate of thermohalophilic bacteria CAT3.4 with several *Bacillus* species as reference. This research uses the maximum likelihood algorithm with the consideration that this algorithm has a higher level of validation than other algorithms.

Muzuni *at al.*[19] explains that the maximum likelihood algorithm calculates the tree with the best sequence variation count. The maximum likelihood algorithm considers, for each phylogenetic tree, the number of sequence changes or mutations that have occurred that provide sequence variation. The maximum likelihood algorithm displays additional opportunities to evaluate trees with variations in the mutation rate in different lineages. A phylogenetic tree based on the nucleotide sequence of type 2 L-asparaginase is presented in Fig. 5.

Figure 5 shows the level of relationship between the thermohalophilic bacterial isolate CAT3.4 as a sample and 9 comparison bacteria from the genus Bacillus. The phylogenetic tree forms 2 clades. The first clade can be called the *Bacillus subtilis* clade because it consists of strains of members of the *Bacillus* genus, namely *Bacillus subtilis* strain GOT9, *Bacillus subtilis* strain SRCM103773, *Bacillus subtilis* subsp. subtilis str. 168 and *Bacillus subtilis* strain B11-06 and the thermohalophilic bacterial isolate CAT3.4 joined this clade. Meanwhile, the second clade consists of *Bacillus licheniformis* strain 14ADL4, *Bacillus halotolerans* strain F41-3, *Bacillus halotolerans* strain XH-1, *Bacillus pumilus* strain TUAT1 and *Bacillus pumilus* strain MTCC B6033.

The thermohalophilic bacterial isolate CAT3.4 forms a clade with the *Bacillus subtilis* group and is in the same subclade with the GOT9 strain *Bacillus subtilis* with a bootstrap value of 96%. These results show that the thermohalophilic bacterial isolate CAT3.4 is thought to have a closer kinship with the *Bacillus subtilis* group than other *Bacillus* species as comparisons. The results of this study were supported by previous studies that isolates of thermohalophilic bacteria CAT3.4 have been identified as phonetically similar to the *Bacillus subtilis* group[8]. In addition, the bootstrap value of the branching isolates of thermohalophilic bacteria CAT3.4 and the *Bacillus subtilis* group was $\geq$50%. A high bootstrap value indicates that the group composition is consistent and the chance of changing the group composition is meager. A high bootstrap value on a branch and the formation of the same node can indicate the level of accuracy of branching in a phylogenetic tree[8,19].

Analysis of the restriction enzyme cutting site type 2 L-asparaginase: Restriction enzyme cutting site analysis was performed using the BioEdit program. Buckhout-White *et al.*[20] explained that, restriction enzymes are enzymes coded by bacteria to recognize short nucleotide sequences and cut DNA only at the cutting sites. The results of the restriction enzyme site analysis were presented in Table 2.

Table 2: Cutting sites for various restriction enzymes using the BioEdit program

Restriction enzyme	Recognition site	Isolate CAT3.4	*B. subtilis* str. B11-06	*B. subtilis* str. GOT9	*B. subtilis* str. SRCM103773	*B. subtilis* str. 168	*B. halotolerans* str. F41-3	*B. halotoleran* str. XH-1	*B. pumilus* str. TUATI	*B. licheniformis* str. 14ADL4	*B. velezensis* str. UCMB5044
*Afe*I	AGC'GCT(*)	523	523	523	523	523	-	-	-	-	582
*Age*I	A'SSGG_T(**)	478	478	478	478	478	484	484	-	-	-
***BsrG*I**	T'GTAC_A(**)	543	543	543	543	543	-	-	-	837	-
***Dra*I**	TTT'AAA(*)	670	670	670	670	670	676	676	369	206	658
		1023	1023	1023	1023	1023	-	-	399	-	-
***Eco*RV**	GAT'ATC(*)	712	712	712	712	712	-	-	820	220	-
***Hind*III**	A'AGCT_T(**)	1107	1107	1107	1107	1107	-	-	-	208	-
*Hpa*I	GTT'AAC(*)	313	313	313	313	313	319	319	-	-	-
***Hpy*CH4IV**	A'CG_T'(**)	291	291	291	291	291	321	321	6	405	197
		315	315	315	315	315	639	639	333	453	714
									966	492	
*Mfe*I	C'AATT_G(**)	699	699	699	699	699	-	-	-	-	171
											687
***Ssp*I**	AAT'ATT(*)	331	331	331	331	331	171	-	175	-	-
		345	345	345	345	345	1130	-	-	-	-
*Swa*I	ATTT'AAAT(*)	670	670	670	670	670	-	-	-	-	658
***Taq*I**	ACGT(***)	294	294	294	294	294	324	324	9	408	200
		318	318	318	318	318	642	642	336	456	717
									969	495	

*DNA fragments has a blunt end, **DNA fragments has a sticky/cohesive end at 5', ***DNA fragments has a sticky/cohesive end 3' and Bold enzymes identifier for *Bacillus subtilis*

The analysis results in Table 2 show that the type 2 L-asparaginase gene has a restriction enzyme recognition site that is palindromic and consists of 4 to 8 base pairs. The same restriction enzyme can occupy different positions in the gene sequence that encodes type 2 L-asparaginase in isolates of thermohalophilic bacteria CAT3.4 and reference bacteria. The gene encoding type 2 L-asparaginase in the thermohalophilic bacterial isolate CAT3.4 has the same position as all reference *Bacillus subtilis* species and differs from other *Bacillus* species. Differences in the cutting pattern of the same type of restriction enzyme indicate nucleotide variations from thermohalophilic bacteria CAT3.4 isolates and reference bacteria encoding the L-asparaginase gene type 2. The restriction enzyme has three cutting ends: Blunt end, sticky/cohesive end at 5' and sticky/cohesive end at 3'. Restriction enzymes with blunt ends are *Afe*I, *Dra*I, *Eco*RV, *Hpa*I, *Ssp*I and *Swa*I, the cutting ends of the sticky/cohesive end at 5' were *Age*I, *BsrG*I, *Hind*III and *Mfe*I and the cutting end of the sticky/cohesive end at 3' is *Taq*I.

Table 2 shown that, the restriction enzymes *BsrG*I, *Dra*I, *Eco*RV, *Hind*III, *Hpy*CH4IV, *Ssp*I and *Taq*I can be used as molecular markers for the L-asparaginase gene type 2 for *Bacillus subtilis* species. The seven restriction enzymes have the same position in the comparison *Bacillus subtilis* species and have different cutting positions in other *Bacillus* species. Schneider and Jejjala[21] reported that certain restriction enzymes can cut DNA at different positions in one species or between different species due to the influence of mutations in these organisms. Differences in nucleotide sequences are why restriction enzyme cleavage sites can be used to identify certain organisms.

Analysis of the amino acid sequence of the type 2 L-asparaginase gene: Translation of nucleotide sequences into amino acid sequences was carried out using the Expasy translate tools. Translation of the nucleotide sequences CAT3.4 thermohalophilic bacterial isolates resulted in 375 amino acids (Fig. 6).

The amino acid composition of the type 2 L-asparaginase gene sequence obtained from the expasy site, namely alanine 9.5%, arginine 2.4%, asparagine 5.9%, aspartate 7.0%, cysteine 0.3%, glutamine 2.7%, glutamic acid 5.1%, histidine 0.8%, isoleucine 5.4%, leucine 7.8%, lysine 8.1%, methionine 1.6%, phenylalanine 3.0%, proline 3.2%, serine 7.8%, threonine 9.5%, tyrosine 3.0% and valine 8.9%. The sequence of amino acids in Figure 6 shows that type 2 L-asparaginase comprises two types of amino acids based on their polarity. The non-polar amino acids that make type 2 L-asparaginase are alanine, arginine, isoleucine, leucine, methionine, phenylalanine, proline, serine, tyrosine and valine, with a total percentage of 42.4%. Meanwhile, polar amino acids, namely cysteine, glutamic acid, histidine, arginine, serine, lysine, aspartic acid, glutamine, threonine and asparagine, have a total percentage of 57.6%.

Based on UniProt site data, the type 2 L-Asparaginase enzyme *Bacillus subtilis* (UniProtKB-O34482 (ASPG2_BACSU)) contains two isoenzymes, namely type 1 L-asparaginase and type 2, where both enzymes have different nucleotide and amino acid structures but catalyze substrates. The same (L-asparagine). Type 1 L-asparaginase secretion is constitutive in the cytoplasm[22]. Meanwhile, type 2 L-asparaginase is secreted in the periplasmic space and has a higher affinity for hydrolyzing L-asparagine[23].

```
1    M K K Q R M L V L F T A L L F V F T G C S H S P E T K E S P   30
31   K E K T Q T Q K V S S A S A S E K K D L P N I R I L A T G G   60
61   T I A G A D Q S K T S T T E Y K A G V V G V E S L I E A V P   90
91   E M K D I A N V G G E Q I V N V G S T N I D N K I L L K L A   120
121  K R I N D L L A S D D V D G I V V T H G T D T L E E T A Y F   150
151  L N L T V K S D K P V V I V G S M R P S T A I S A D G P S N   180
181  L Y N A V K V A G A P E A K G K G T L V V L N D R I A S A R   210
211  Y V T K T N T T T T D T F K S E E M G F V G T I A D D I Y F   240
241  N N E I T R K H T K D T D F S V S N L D E L P Q V D I I Y G   270
271  Y Q N D G S Y L F D A A V K A G A K G I V F A G S G N G S L   300
301  S D A A E K G A D S A V K K G V T V V R S T R T G N G V V T   330
331  P N Q D Y A E K D L L A S N S L N P Q K A R M L L M L A L T   360
361  K T N D P Q K I Q A Y F N E Y                               375
```

Fig. 6: Amino acid sequence of the gene encoding L-asparaginase type 2 in isolates of thermohalophilic bacteria CAT3.4
MKKQRMLVLFTALLFVFTG: Signal peptide, T: Active site and TD: Substrate binding site

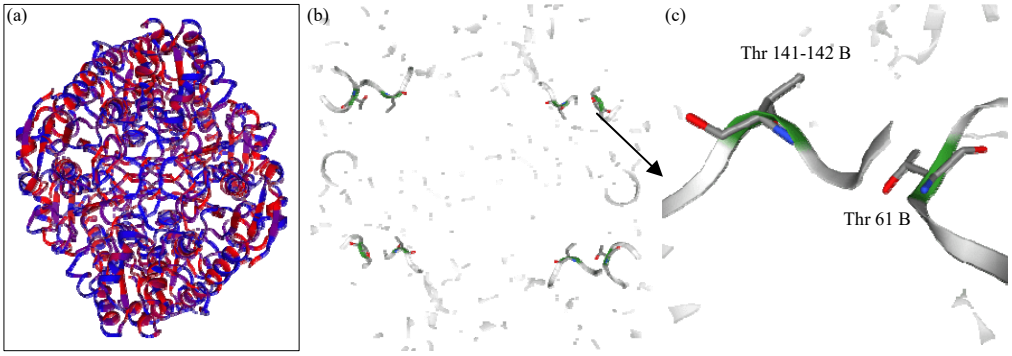

Fig. 7(a-c): 3D structure of L-asparaginase type 2 in *Bacillus subtilis*, obtained from the Swiss Model Expasy site, (a) 3D structure of L-asparaginase type 2, (b): Homotetramer (there are 4 active sites) and (c) Active site (Thr 61 B) and binding site (Thr 141-142 B)

The amino acid sequence of the L-asparaginase gene in Figure 6 was a signal peptide that plays an important role in signalling when cells are in a state of nitrogen crisis and carrying type 2 L-asparaginase to the periplasmic for secretion. The N-terminal peptide signal is a sequence of amino acids that directs the protein to enter the secretory pathway to be secreted into the periplasm, the extracellular environment or the cell wall[23].

Bacillus subtilis, which was used as a comparator in this study, has the same amino acid sequence, so the data obtained from the Swiss Model Expasy website can be used as a reference to see the 3D structure of the type 2 L-asparaginase enzyme from *Bacillus subtilis*. type 2 L-asparaginase has a homotetramer structure consisting of four identical subunits, each with an active site[22,24]. Related to this structure, type 2 L-asparaginase has a high affinity for hydrolyzing asparagine. According to Lubkowski and Wlodawer[25], based on its conformation and affinity level, the type 2 L-asparaginase enzyme has a homotetramer conformation, so it has a high affinity for hydrolyzing asparagine. The type 2 L-asparaginase enzyme is secreted in response to a lack of nitrogen in cells. The 3D structure of the type 2 L-asparaginase enzyme was presented in Fig. 7a-c.

Figure 7 shows the presence of additional domains, such as the active site at sequence 61 in the form of the amino acid threonine (T) and the substrate binding site at sequence 141-142 in the form of the amino acids threonine (T) and aspartate (D). In general, the binding site is where the substrate attaches to the active site in the form of the amino acid threonine, which plays a role in the hydrolysis of asparagine into aspartate and ammonia.

Next, phylogenetic tree construction was carried out based on the amino acid sequence of the type 2 L-asparaginase gene. The phylogenetic tree was constructed using the MEGA X program and the maximum likelihood algorithm. The results of the analysis of the amino acid phylogenetic tree construction were presented in Fig. 8.

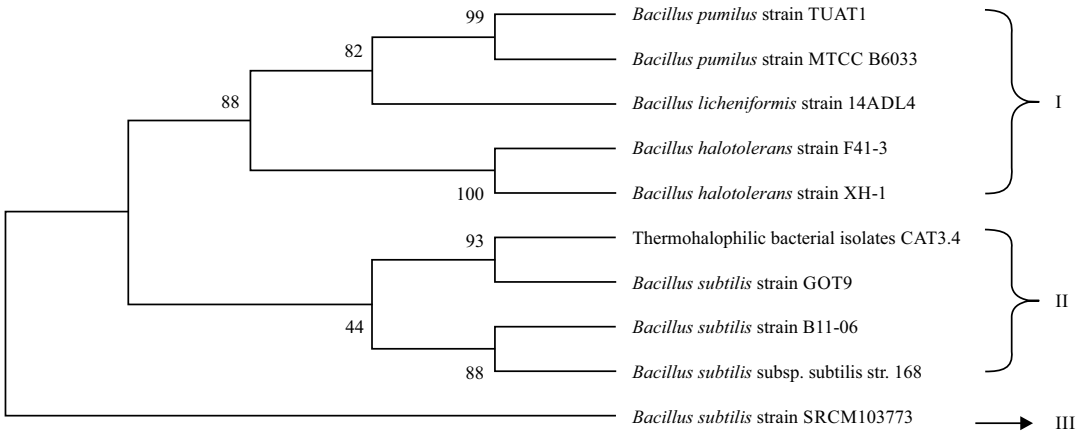

Fig. 8: Phylogenetic tree based on the amino acid sequence of the L-asparaginase type 2 coding gene fragment of CAT 3.4 thermohalophilic bacterial isolates using the MEGAX program

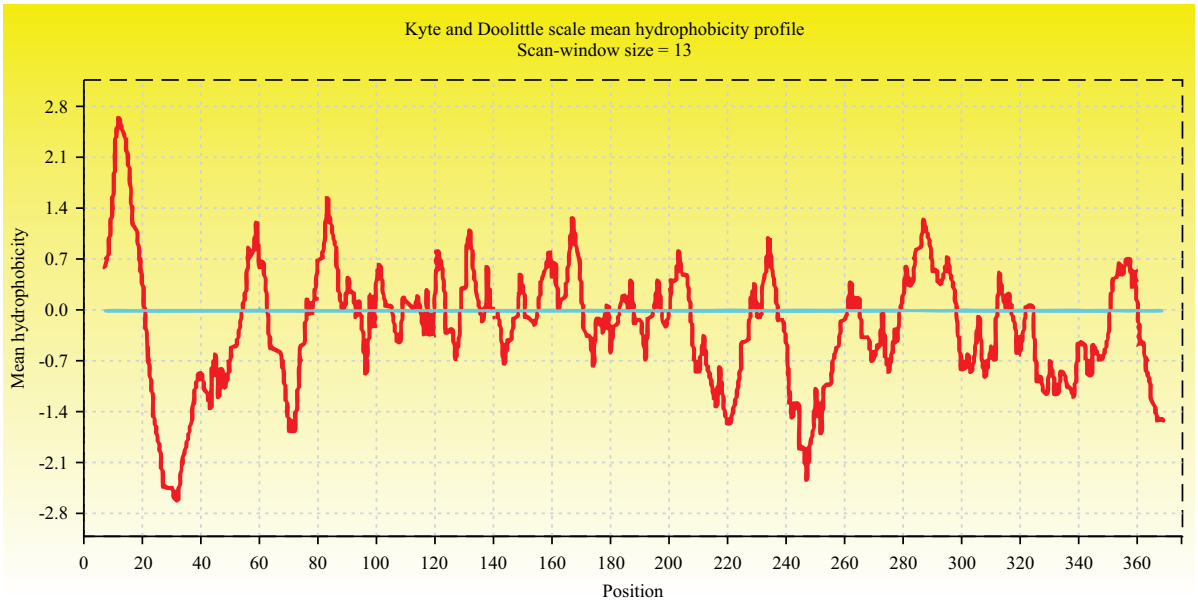

Fig. 9: Hydrophobicity profile of the gene encoding L-asparaginase type 2 isolate CAT3.4 thermohalophilic bacteria

Based on Fig. 8, the phylogenetic tree shows that the thermohalophilic bacteria isolate CAT3.4 is in the same clade as the *Bacillus subtilis* group. These results show a branching pattern almost the same as a phylogenetic tree based on nucleotide sequences (Fig. 5). The phylogenetic tree based on nucleotide and amino acid sequences shows the same branching, except that in the phylogenetic tree based on amino acids, *Bacillus subtilis* strain SRCM103773 leaves the group to form its branching. The release of *Bacillus subtilis* strain SRCM103773 from the group was caused by a substitution mutation in the 158th nucleotide of the type 2 L-asparaginase gene. Mutations that occur can affect the results of amino acid translation because they will also change, resulting in a different amino acid composition.

Hydrophobicity analysis of the type 2 L-asparaginase enzyme: Hydrophobicity analysis of an amino acid can be determined using the BioEdit program. The hydrophobicity profile can illustrate that an amino acid is hydrophilic if the peak is below the 0 line and an amino acid is hydrophobic if the peak is above the 0 line. The hydrophobicity analysis results curve was presented in Fig. 9.

Based on the results of the Kyte and Doolittle hydrophobicity analysis in Fig. 9, the amino acid sequence of the gene encoding L-asparaginase from the thermohalophilic bacterial isolate CAT3.4 is in the hydrophilic and hydrophobic regions. However, it is predominantly in the hydrophilic region where the dominant red protein peak is oriented in the hydrophilic region (0 to -2.5). The hydrophilic condition of the gene encoding type 2 L-asparaginase is influenced by its constituent amino acids, which are dominated by polar amino acids (Fig. 6). In addition, type 2 L-asparaginase is secreted in the periplasmic space, where the periplasmic space is between the outer membrane and the inner membrane of the bacteria, which is filled with a thick, polar fluid. According to Dumina *et al.*[26], type 2 L-asparaginase exists under anaerobic conditions in the periplasmic space of the two layers of the bacterial membrane and has a higher specificity for hydrolyzing asparagine.

This research can determine the character of the gene encoding type 2 L-asparaginase, which includes gene size, nucleotide and amino acid similarity, restriction enzyme cutting sites and amino acid structure. The gene obtained can be applied in the health sector as a chemotherapy agent and in the food sector, where it can reduce food acrylamide levels. This research is only limited to obtaining the gene encoding type 2 L-asparaginase and characterizing it. Information about gene characteristics can be used to develop strategies for cloning genes into expression vectors to produce enzymes in host cells.

CONCLUSION

Characteristics of the gene encoding the enzyme type 2 L-asparaginase isolate the most expensive bacterium CAT3.4 has a nucleotide sequence of 1128 bp in size with the closest degree of kinship to *Bacillus subtilis* strain GOT9 based on a phylogenetic tree with a bootstrap value of 96% and a similarity index from BLASTn analysis with an identity value of 100%. Analysis of the restriction enzyme cutting sites showed that seven types of restriction enzymes could be used as markers for the gene coding for type 2 L-asparaginase enzymes, namely *Bsr*GI, *Dra*I, *Eco*RV, *Hind*III, *Hpy*CH4IV, *Ssp*I and *Taq*I. The nucleotide sequence of the type 2 L-asparaginase gene is then translated to produce 375 amino acids, which are polar and non-polar and are predominantly in the hydrophilic region.

SIGNIFICANCE STATEMENT

This research has isolated and characterized the gene encoding type 2 L-asparaginase from the thermohalophilic bacteria isolate CAT3.4. This enzyme could be applied as an inhibitor of the development of leukemia and as a reducer of acrylamide levels in food. Therefore, this study will help researchers find the gene that produces the type 2 L-asparaginase enzyme, which inhibits leukemia and reduces acrylamide levels. In this study, the characteristics of the gene encoding the type 2 L-asparaginase enzyme were found in the thermohalophilic bacterial isolate CAT3.4, which has a nucleotide sequence of 1128 bp and the closest relationship to *Bacillus* subtilis strain GOT9.

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