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ARAŞTIRMA MAKALESİ RESEARCH PAPER

Evaluation of the Potential of Thermophilic *Anoxybacteroides amylolyticum* GCHI to Form BH4 Main-Substrate [*]

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Abstract: In this study, the cloning, expression and biochemical characterization of GTP cyclohydrolase I (GCHI), the first enzyme involved in phenylketonuria metabolism, from the thermophilic bacterium *Anoxybacteroides amylolyticum* were investigated. The *gchI* gene, identified by PCR from *A. amylolyticum* DSM 15939^T, is a single open reading frame of 555 bp encoding a total of 184 amino acids. Expression of the enzyme, which catalyzes the conversion of GTP to dihydroneopterin triphosphate, was cloned into pET28a(+) and carried out in *E. coli* BL21(DE3) cells in the presence of 1 mM IPTG. A Ni-NTA affinity column was used for enzyme purification. Characterization studies revealed that the enzyme had a V_{max} value of $6.9x10^{-5} \pm 2.3x10^{-4}$ µM/min/mg protein and a K_m value of 7.0 ± 1.4 mM. The enzyme showed the best activity at an optimal pH of 7.2 at an optimum temperature of 70°C. It was shown that thermophilic *A. amylolyticum* GCHI can be used in the first step of GTP conversion reactions under *in vitro* conditions as an alternative for the chemical synthesis of BH4 used in the treatment of phenylketonuria.

Keywords: *Anoxybacteroides amylolyticum*, BH4, GCHI, GTP, neopterin.

Termofilik *Anoxybacteroides amylolyticum* GCHI'nin BH4 Ana Substratını Oluşturma Potansiyelinin Değerlendirilmesi

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Öz: Bu çalışmada, fenilketonüri metabolizmasında rol oynayan ilk enzim olan GTP siklohidrolaz I (GCHI)'in, termofilik bakteri *Anoxybacteroides amylolyticum* klonlanması, ekspresyonu ve biyokimyasal karakterizasyonu araştırılmıştır. *A. amylolyticum* DSM 15939^T'dan PCR ile tanımlanan *gchI* geni, 555 bp boyutunda toplam 184 amino asidi kodlayan tek bir açık okuma çerçevesidir. GTP'nin dihidroneopterin trifosfata dönüşümünü katalize eden enzimin ekspresyonu, pET28a(+) içine klonlanarak 1 mM IPTG varlığında *E. coli* BL21(DE3) hücrelerinde gerçekleştirildi. Enzimin saflaştırılması için Ni-NTA afinite kolonu kullanıldı. Karakterizasyon çalışmaları enzimin, V_{max} değerinin $6,9x10^{-5} \pm 2,3x10^{-4}$ µM/dk/mg protein ve K_m değerinin 7,0 ± 1,4 mM olduğunu ortaya koymuştur. Enzim, optimum 70°C sıcaklıkta 7,2 optimal pH'da en iyi aktiviteyi gösterdi. Termofilik *A. amylolyticum* GCHI'nin, fenilketonüri tedavisinde kullanılan BH4'ün kimyasal sentezine alternatif olarak, *in vitro* koşullar altında GTP'nin dönüşüm reaksiyonlarının ilk basamağında kullanılabileceği gösterilmiştir.

Anahtar Kelimeler: *Anoxybacteroides amylolyticum*, BH4, GCHI, GTP, neopterin.

INTRODUCTION

GTP cyclohydrolase I (GTPCH1, GCHI) [EC 3.5.4.16] is a primary enzyme involved in the biosynthesis of the BH4 (tetrabiopterin) cofactor in humans. A common reaction step in the formation of this cofactor, which is required in disorders such as phenylketonuria, is the formation of dihydroneopterin triphosphate (NH_2TP, 6-D-threo-1′,2′,3′-hydroxypropyl-7,8-dihydroneopterin 3′-triphosphate) from GTP (Figure 1). This process proceeds in a multistep manner, beginning with the cleavage of the purine base and ending with the formation of the pyrazine ring (Tanaka et al., 2005). Because of its metabolic importance in humans, the GCHI enzyme has been studied in prokaryotic sources and has also been of interest in bacteria due to its role in folic acid synthesis. Similarly, due to its role in BH4 formation, many researchers have used prokaryotic GCHI in the first stage for the conversion of BH4 starting from GTP as an alternative to synthetic BH4 production and have addressed not only kinetic but also structural studies of the enzyme. Phenylketonuria (PKU), the most important of the BH4-related diseases, is an autosomal recessive disease and occurs when the liver enzyme

[*] *This study was produced from Müride KALEMCİ's the master thesis.*

[*] *Bu makale, Müride KALEMCİ'nin yüksek lisans tezinden üretilmiştir.*

phenylalanine hydroxylase (PAH) is not synthesized or is not sufficiently active, resulting in patients being unable to metabolize phenylalanine. This inhibits the conversion of phenylalanine to tyrosine (Kazancı, 2018). With a prevalence of 1 in 4,500 in our country whereas 1 in 10,000 in Europe, 250-300 children are born with PKU each year. One in every 20-25 people in Türkiye carries the disease and consanguineous marriages are common, contributing to its high prevalence (Koyuncu, 2010). This disease also occurs due to the deficiency of enzymes involved in the synthesis or reduction of coenzyme BH4. The most significant symptoms of this disease are severe mental retardation and neurological and developmental disorders.

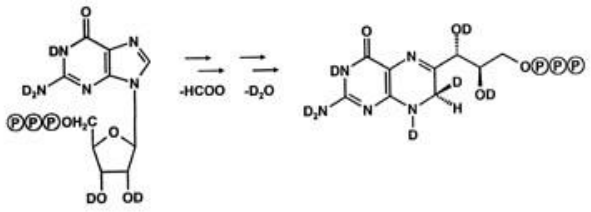

Figure 1. Reaction pathway of GCH-I (revised form Bracher et al., 1998).

Defects in the enzymes dihydropteridine reductase, GTP cyclohydrolase-I (*gch*I), pterin-4-8-carbinolamine dehydratase, and 6-pyrrolyl tetrahydropterin synthase (*ptps*) can be identified in the disruption of BH4 metabolism and can affect the liver, kidneys, brain, and blood tissue (Beris et al., 2022; Blau and Burgard, 2005). The cost of chemical synthesis of BH4 is quite high due to the difficulty of the chemical synthesis, the complexity of the methods used, the use of many synthetic steps and the expense of the chemicals and processes to be used. Due to these difficulties, an alternative to BH4 production can be provided biologically by recombinant cloning of bacterial genes. Thus, BH4 production can be done in humans using bacterial homologs of the enzymes involved in this pathway. Cells can obtain BH4 from GTP as a precursor in the *de novo* biosynthesis pathway and from dihydropterins already present in the medium in the Salvage pathway. The *de novo* biosynthesis mentioned here occurs through the sequential reaction of three main enzymes known as GCHI, PTPS, and SPR, and this is valid both *in vivo* and *in vitro* (Beris et al., 2022; Çelik, 2018; Moens & Kass, 2006). Treatment options for the disease may include phenylalanine restriction in a tyrosine-supplemented diet or BH4 administration. Therefore, BH4 synthesis has great importance. As an alternative to the difficulties in chemical synthesis of BH4, enzymatic synthesis approaches using enzymes responsible for BH4 conversion are gaining increasing importance. GCH1, the critical enzyme in the first step of synthesis, has been purified from various species, and its basic biochemical properties, including the thermal and pH dependence of its activities, have been studied. However, the identification of functional groups of GCH1 has been problematic, in part due to the instability of the enzyme (Tanaka et al., 2005). Therefore, the search for thermophilic sources of the enzyme as an alternative to mesophilic sources is of interest.

The first study of an alternative to the chemical synthesis of BH4 was conducted by Yamamoto et al. (2003). In this study, BH4 conversion was investigated by cloning the *ptps* and *spr* genes obtained from mice and the *gchI* gene analog (*mtr*A) obtained from *B. subtilis*. In a similar study, Beris et al. (2022) characterized the first enzyme of the pathway by recombinantly cloning the *gchI* gene from the thermophilic *Anoxybacillus flavithermus* DSM 2641^T bacterium. Today, enzymes obtained from thermophilic organisms are frequently used in many biotechnological and industrial applications. The reasons such as the fact that the reactions take place at high temperatures, the reduction of the risk of general mesophilic contamination of the environment, the absence of undesirable by-product formation, the increase in the solubility of substrates and products, and the increase in stability due to the formation of preferred equilibrium changes in endothermic reactions constitute the attractive elements of the preference of these thermophilic enzymes, in demanding industrial applications (Beris et al., 2022; Çelik, 2018; Kalemci, 2020). In this study, we investigated the recombinant cloning and characterization of GCHI from the thermophilic bacterium *Anoxybacteroides amylolyticum* and discussed whether it could be a candidate enzyme for BH4 conversion *in vitro*.

MATERIAL AND METHOD

Microorganisms, Chemicals, and Other Materials: *Anoxybacteroides amylolyticum*, *E. coli* DH5α, and *E. coli* BL21(DE3) bacteria were obtained from our laboratory stocks. Modifying enzymes and substrates were purchased from ThermoScientific (USA). Purification kits for PCR products and DNA restriction fragments were obtained from Wizard® SV Gel and PCR Clean–Up System (Promega Co., Medison, USA). Plasmid and genomic DNA isolation kits were purchased from Thermo Sci. and Promega Co. (GeneJet Plasmid Miniprep Kit and Wizard® Genomic DNA Purification Kit, USA, respectively). All other chemicals and reagents were obtained from Sigma & Aldrich Inc. (St. Louis, MO, USA) and Merck GmbH (Germany). All primers for PCR were purchased from Macrogen Inc. synthesized by (Amsterdam, Netherlands).

Cloning of AmygchI gene: Genomic DNA isolation from *A. amylolyticum* was performed with the Promega Wizard Genomic DNA Purification Kit according to the manufacturer's protocol. To obtain the *amygchI* gene by PCR, the *gchI* gene designated as Accession Number CP015438 was introduced into the bacteria using forward (5'-CCATGGTAAACTACGAACAAATCG–3') primers containing an *Nco*I restriction site (underlined) and reverse

(5'-CTCGAGTCCTTTAATTTAATGAAAGCAC-3') primers containing an *Xho*I restriction site (underlined). PCR was prepared in the final volume of 50 µL, containing 500 ng gDNA, 200 µM dNTP, 50 pmol forward and reverse primer, 1X PCR Buffer, 2 mM $MgCl_2$, and 1 U *Taq* DNA polymerase enzyme. PCR cycle conditions were applied as 5 cycles of pre-denaturation at 95ºC for 2 minutes, denaturation at 94ºC for 90 seconds, annealing at 48ºC for 90 seconds and synthesis at 72ºC for 2 minutes, followed by 35 cycles of denaturation at 94ºC for 1 minute, annealing at 54ºC for 90 seconds, synthesis at 72ºC for 2 minutes and the reaction was completed with the final synthesis at 72ºC for 10 minutes. The obtained amplicon was purified with DNA Clean&Concentrator™ (Zymo Research, USA) kit as recommended by the manufacturer and cloned into pGEM®-T/Easy Vector (Promega Co., USA). The obtained recombinant vector was transferred into *E. coli* DH5α host cells. After verification of the recombinant vector, it was cut with relevant restriction enzymes and cloned into pET28a(+) and introduced into *E. coli* BL21(DE3) for expression. Plasmid verification after isolation by using ZymoPURE™ Plasmid Miniprep kit (Zymo Res., USA) was performed by restriction enzyme digestion, PCR, and DNA sequence analysis.

Expression and Purification of Recombinant Enzyme: One colony was plated in LB broth containing kanamycin (30 µg/mL) and incubated overnight at 37°C at 150 rpm. When the optical density at 600 nm of the starter culture obtained here reached 0.8, cells were induced with 1 mM IPTG. After 4 hours, the pellet of the cultures was obtained by centrifuging at 6000 rpm for 10 min at 4°C. The pellet was washed in 1X PBS, then precipitated again and resuspended in phosphate buffer (pH 7.0). The supernatant taken from the cells disrupted by sonication (30% amplitude, 2 min ON/OFF, 5 min in total) was incubated at 60°C for 30 min to remove mesophilic proteins. The obtained supernatant was purified by His Link™ Protein Purification System (Promega, Medison, WI, USA) according to the manufacturer's protocol. The purified protein was then checked by SDS-PAGE (12% w/v). The protein concentration of the dialyzed sample was then determined according to Bradford methods (Bradford, 1976).

Enzyme Assays: Enzyme activity was performed according to Beris et al. (2022). In this method, the incubation temperature was used as 55°C before determining the biochemical parameters. Briefly, the reaction containing 50 mM Tris HCl pH 8.0, 100 mM KCl, 2 mM $MgCl_2$, 1 mM GTP and 0.2 µM enzyme in a final volume of 100 µL was incubated at 55°C for 1 hour. The reaction was then terminated by adding an oxidation solution containing 1% I_2 and 2% KI in 1N HCl in the dark. The reduction was completed by adding 15µL of 2% ascorbic acid to the reaction. The fluorescence of neopterin triphosphate (NH_2TP), the product formed after the enzymatic reaction, was measured with excitation at 350 nm and emission at 450 nm using a spectrofluorometer (SpectraMax, Molecular Devices, USA). The graph drawn using neopterin as the reaction standard was used.

Biochemical Characterization of AmyGCHI and Determining Its Kinetic Parameters:

Effects of pH and temperature on AmyGCHI activity: The effects of pH and temperature on activity were determined in the pH range of 5.5-9.0 and 37-85°C. MES was used for pH 5.5 and 6.0, potassium phosphate for pH 5.8-8.0, Tris-HCl for pH 7.5-8.8 and glycine-NaOH for pH 9.0. After determining the optimum pH, the reaction was carried out between 37-85°C with 5-degree increments for optimum temperature experiments. Determination of kinetic parameters was determined using main substrate, GTP, in the concentration range ranging from 10 µM to 2 mM under optimized conditions (at pH 7.2 and 70°C). *Km* and V_{max} values of AmyGCHI were calculated using Origin 8.0 program. All reactions were repeated three times.

Determination of inhibitors and activators on enzyme activity: To determine the effect of metals on enzyme activity, experiments were performed using Zn^{2+}, Hg^{2+}, Cu^{2+}, Na^{+}, Ca^{2+}, Fe^{3+}, Al^{3+}, Mn^{2+}, Mg^{2+}, and K^{+} metal ions at 0.5 - 10 mM concentrations. In addition, experiments were performed with SDS, TritonX100, and EDTA compounds at 0.1, 0.2, 0.3, and 1%; 0.5, 1, 2, 5, 10, and 20%; 0.5, 1, 3, 5, and 10 mM respectively. Their relative activities were determined according to the graph drawn using the obtained data. All reactions were repeated three times.

RESULTS AND DISCUSSION

In this study, the characterization of the GCHI enzyme belonging to thermophilic *A. amylolyticum* bacteria was done. The data obtained from the gene encoding the enzyme was obtained using NCBI data obtained from *A. amylolyticum* DSM 15939^T, whose genome analysis had been completed before. In this data, it was found that the primers designed by PCR were a 555 bp gene encoding an 184 amino acid open reading frame sequence as in the original strain. The relevant gene was cloned into the pET28a(+) vector to add 6 histidine residues at the C terminus for ease of purification and was successfully expressed in *E. coli* BL21(DE3) cells. Due to the thermophilic enzymes structure of the enzyme, the obtained cell extract eliminated most of the host cell proteins by heat treatment at 60°C for 30 minutes. Then, the enzyme was purified by Ni-NTA affinity chromatography and dialysis was performed to remove imidazole. SDS-PAGE analysis (12%) showed that the purified enzyme was homogeneous, and its molecular mass was approximately 21 kDa (Figure 2).

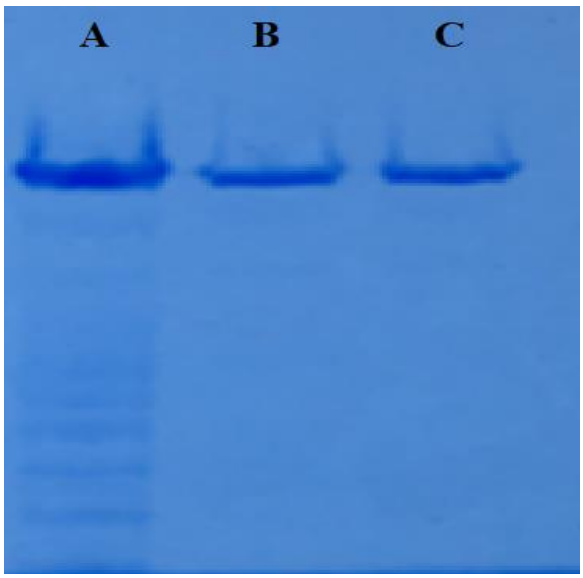

Figure 2. 12% SDS-PAGE profiles of AmyGCHI (A: Crude extract, B: Heat inactivation, and C: Ni-NTA purified enzyme).

Among thermophilic bacteria, AmyGCHI amino acid sequence has 97.83% similarity to *A. flavithermus* WK1 GCHI, 88.04% to *G. thermodenitrificans* strain ID-1, 86.96% to *G. thermoleovorans* strain KCTC 3570, and 86.41% to *G. stearothermophilus* strain 15. As shown in Fig. 3, amino acid modeling was performed using the Escript program (Robert & Gouet, 2014) by comparing the amino acid sequences of mostly thermophilic enzymes with the GCHI (1WUQ) of *Thermus thermophilus* HB8. In this figure, fully conserved amino acids are shown in white letters on a red background. Analysis revealed that ligand-binding pockets are present between 89.-100., 129.-132., and 158.-178. amino acids and are highly conserved. Cys91, His94, and Cys163 represent metal ion binding sites, while His93 and His161 represent enzymatic catalysis sites according to homology of 1WUQ.

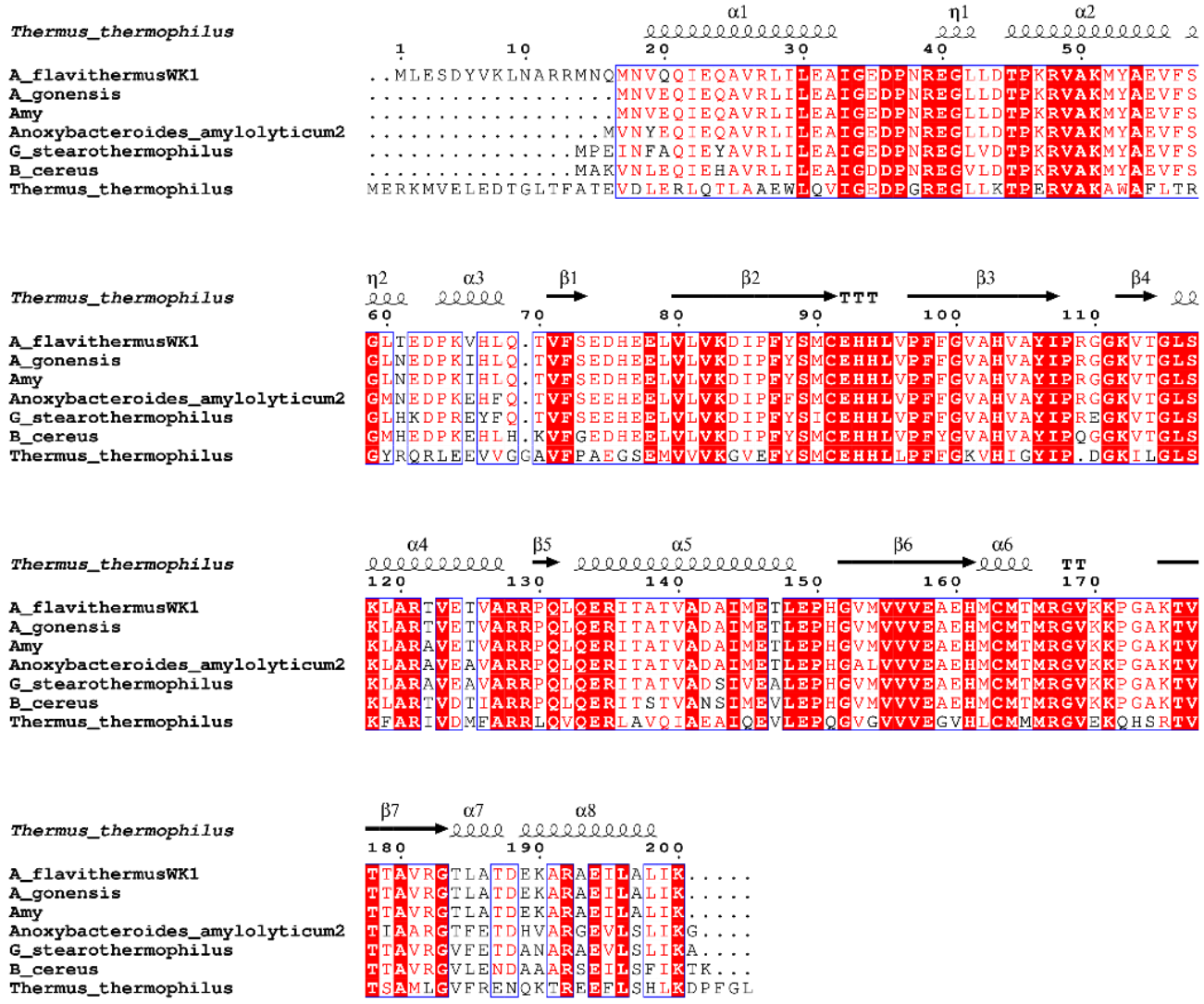

Figure 3. Comparison of GCH1 amino acid sequences from various species. Fully conserved residues are shown in white letters on a red background. Partially conserved residues are shown in red letters. The modeling figure was generated using ENDscript (Robert & Gouet, 2014) based on the *T. thermophilus* HB8 GCHI.

The optimum pH for AmyGCHI is 7.2 with GTP substrate. Under optimal conditions, the activity of the enzyme decreases by an average of 20% between pH 5.5 and 6.5, by 37% at pH 7.5, and by 15% at alkaline pHs (Figure 4a). The optimum activity of GCHIs is 8.0 for *A. flavithermus* DSM 2641^{T} (Beris et al., 2022), *G. sterothermophilus* ATCC 8005 (Suzuki et al., 1979), and B. *subtilis* (De Saizieu et al., 1995), and 8.5 for *Thermus thermophilus* HB8 (Tanaka et al., 2005). The optimum temperature for AmyGCHI was determined at 70°C. At 55°C, the optimum bacterial growth temperature, the enzyme shows 80% of its original activity (Figure 4b). In other studies, we see that the optimum temperature value varies depending on how it functions, namely 42°C for *E. coli* (Yim & Brown, 1976), 37-78°C for *G. sterothermophilus* ATCC 8005 (Suzuki et al., 1979) and 37°C for *B. subtilis* (De Saizieu et al., 1995). We see that AfGCHI from *A. flavithermus* DSM 2641^{T}, which has a high similarity with the structure to the enzyme, is active at 65°C (Beris et al., 2022).

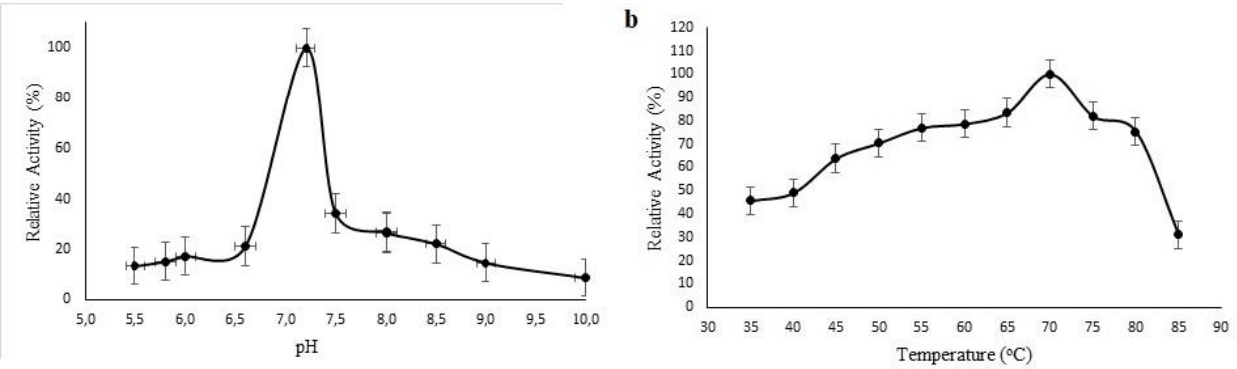

Figure 4. a. Determination of optimal pH of AmyGCHI, **b.** Determination of optimal temperature of AmyGCHI.

The *Vmax* and *Km* of the enzyme were calculated as 6.9 x 10^{-5} ± 2.3 nM/min/mg protein and 7.0 ± 1.4 µM for GTP, the main substrate, in the range of 0.001 to 2 mM (Figure 5). When the *Km* values were compared with the literature, the *Km* values of *A. flavithermus* DSM 2641^{T} were 243 ± 23.25 µM (Beris et al., 2022), 0.0023-0.0053 µM for *Thermotoga maritima* MSB8 (El Yocoubi et al., 2006), 2.9 µM for *E. coli* (Yim & Brown, 1976), 4.2 µM for *Thermus thermophilus* HB8 (Tanaka et al., 2005), 4.0 µM for *B. subtilis* (De Saizieu et al., 1995), 80 µM for *Streptomyces tubercidicus* ATCC 25502 (Yo et al., 1998) and 980 µM for *Geobacillus stearothermophilus* ATCC 8005 (Suzuki et al., 1979). As is well known, the *Km* value of an enzyme decreases as its affinity for its substrate increases. According to literature data, the *Km* value of AmyGCHI is quite good for the GTP substrate, especially when compared to GTPCHI enzymes from various studied sources, especially thermophilic enzymes. This suggests that the enzyme has potential for improvement.

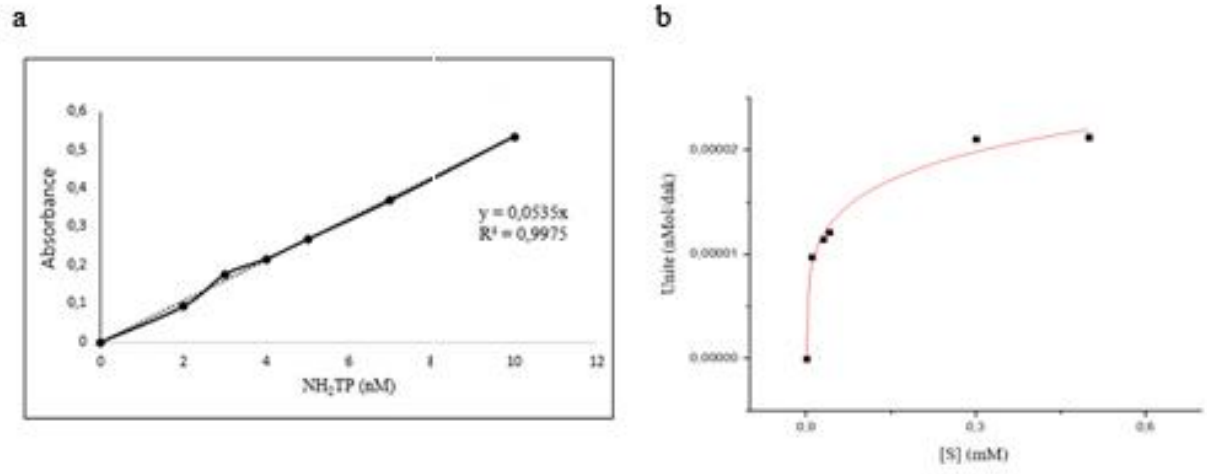

Figure 5. Lineweaver-Burk plots of AmyGCHI under optimal conditions.

Among the metal ions used against enzyme activity, K^{+} ion was found to be 100% effective at a concentration of 5 mM, but reduced activity by half at approximately 27 mM and completely inhibited it at 50 mM. It was determined that Mg^{2+} and Zn^{2+} at 2 mM, Ca^{2+} and Cu^{2+} at 1.5 mM, Fe^{3+} at 3 mM, and Na^{+} at 5 mM

contributed most effectively to enzyme activity. Mg^{2+} at 5 mM and Fe^{3+} at 7 mM were found to inhibit activity. The overall effects of the metal ions studied were evaluated based on relative activity. It was found that the relative activity of EDTA at 1 mM concentration was 35%, inhibition was observed at other concentrations, and SDS inhibited enzyme activity after a concentration of 0.2%. According to BRENDA data, it is seen that *Bacillus subtilis* subsp. *subtilis* str. 168 exhibits 75% activity in Fe^{3+} at 1 mM, 43% in Mg^{2+} at 0.1 mM, 14% in Zn^{2+} at 0.5 mM, 24% in Co^{2+} at 0.1 mM, but no activity in Cu^{2+} and Ca^{2+} (Sankaran et al., 2009). In *Methanocaldococcus jannaschii* MJ0775, it was shown that 2 mM Mn^{2+} stimulated the activity better than Fe^{2+} (Grochowski et al., 2007).

CONCLUSION

In conclusion, it is crucial to develop an alternative to chemical synthesis of BH4, a cofactor and active ingredient used in the treatment of phenylketonuria, a disease with a high prevalence in our country, through enzymatic conversion. Enzymes obtained from mesophilic sources are affected by environmental and chemical reaction conditions, and due to their thermodynamic properties, the use of thermophilic enzymes as an alternative will eliminate such problems. Therefore, thermophilic enzymes are preferred in industrial applications due to their thermophilic nature (Andrade et al., 1999; Hartley et al., 2000; Taylor et al., 2004; Hızal, 2013). This study demonstrates that the human homologue of the GCHI enzyme can be cloned from a thermophilic bacterium, thus enabling the production of NH_2TP without chemical synthesis. Similarly, by cloning and characterizing other enzymes and determining their reaction parameters, active ingredient production can be achieved through enzymatic synthesis.

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