



## Research article

Isolation, expression, and characterization of raw starch degrading  $\alpha$ -amylase from a marine lake *Bacillus megaterium* NL3Sofi Siti Shofiyah<sup>a,b</sup>, Dewi Yuliani<sup>a</sup>, Nurul Widya<sup>a</sup>, Fean D. Sarian<sup>a</sup>, Fernita Puspasari<sup>a</sup>, Ocky Karna Radjasa<sup>c,d</sup>, Ihsanawati<sup>a</sup>, Dessy Natalia<sup>a,e,\*</sup><sup>a</sup> Biochemistry Research Division, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Jl. Ganesha No. 10, Bandung, 40132, Indonesia<sup>b</sup> Marine Science Program Study, Faculty of Marine Science, OSO University, Pontianak, 78113, Indonesia<sup>c</sup> Center for Tropical Coastal and Marine Studies, Diponegoro University, Widya Puraya, Semarang, 50275, Indonesia<sup>d</sup> Indonesian Institute of Sciences, Gatot Subroto 10, Jakarta, Indonesia<sup>e</sup> University Center of Excellence for Nutraceuticals, Biosciences and Biotechnology Research Center, Bandung, Indonesia

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## ABSTRACT

A land-locked marine lake Kakaban with its significant ecological parameters provides a unique habitat for bacteria with novel biotechnology potential that uses a diverse array of catalytic agents, including  $\alpha$ -amylase. Aiming at the isolation of raw starch degrading  $\alpha$ -amylase from marine biodiversity, a gene encoding BmaN2 from a sea anemone associated bacterium *Bacillus megaterium* NL3 was cloned and expressed in *Escherichia coli* ArcticExpress (DE3). It comprises an open reading frame of 1,563 nucleotides encoding BmaN2 of 520 amino acids and belongs to the glycoside hydrolase family 13 subfamily 36 (GH13.36). This  $\alpha$ -amylase has a maximum activity at pH 6.0 and 60 °C with a specific activity of 28.7 U mg<sup>-1</sup>. The BmaN2 activity is enhanced strongly by Ca<sup>2+</sup> but inhibited by EDTA. BmaN2 also exhibits high catalytic efficiency on soluble starch with  $k_{cat}/K_M$  value of 14.1 mL mg<sup>-1</sup> s<sup>-1</sup>. Despite no additional starch-binding domain, BmaN2 is able to hydrolyze various raw starches, such as wheat, corn, cassava, potato, rice, sago, and canna, in which granular wheat is the preferred substrate for BmaN2. These characteristics indicate that BmaN2 is a promising raw starch degrading enzyme within the subfamily GH13.36.

## 1. Introduction

Starch is the second most abundant renewable carbohydrate that functions as energy storage in plants. Plants produce starch as densely packed semi-crystalline granules. Disruption of the semi-crystalline granules through the gelatinization process commonly performed at high temperatures is required prior to starch hydrolysis chemically and/or enzymatically [1].

$\alpha$ -Amylase (EC 3.2.1.1) is an endo-acting enzyme that hydrolyzes  $\alpha$ -1,4-glycosidic bonds of starch producing linear and branch oligosaccharides. It belongs to the family of glycoside hydrolase 13 (GH13), which is the largest group of  $\alpha$ -amylases. Additionally, some  $\alpha$ -amylases are classified into GH57, GH119, and GH126, according to the Carbohydrate-Active enZymes (CAZy) database [2, 3].  $\alpha$ -Amylases have enormous market demands and many applications in the industry [1]. Therefore, considerable efforts have been made to isolate new

$\alpha$ -amylases with unique properties, such as a raw starch degrading  $\alpha$ -amylase (RSDA).

RSDAs are able to degrade raw starch granules below the gelatinization temperature and thus considered to be environmentally friendly. Moreover, the application of RSDAs would simplify the starch processing steps and hence would reduce the production cost of starch-based compounds [4]. The capability of RSDAs in hydrolyzing starch granules is attributed to the presence of an additional starch binding domain (SBD), separated from the catalytic domain, and/or surface binding site (SBS) [5, 6]. RSDAs without SBD could have similar activity in the hydrolysis of concentrated raw starch suspension [6]. Even though research on RSDAs has increased rapidly, the exploration of RSDAs from marine microorganisms is still limited.

Two wild-type RSDAs from marine microorganisms *Bacillus subtilis* S8-1 [7] and *Bacillus sp.* ALSHL3 [8] have been reported. Other studies showed that a recombinant  $\alpha$ -amylase from the marine metagenomic library, AmyP containing natural SBD, can digest several raw starches [9,

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10]. Interestingly, BqA from *Bacillus aquimaris* MKSC 6.2 [11] and AmyASS from *Aeromonas salmonicida* ssp. *Salmonicida* [12] are capable of hydrolyzing raw starches without SBD.

Indonesia, as one of the megadiversity countries, assures the large biodiversity of marine invertebrates, most of which remain to be studied. Since marine diversity also reflects chemical diversity, the isolation of the under-exploited symbiotic bacteria from Indonesian invertebrates offers an excellent opportunity for discovering novel enzymes, including  $\alpha$ -amylase. A unique marine lake environment is located on Kakaban Island, East Kalimantan, Indonesia. It was thought to be formed for a thousand years in which seawater was trapped, turning the area. Rainwater and groundwater mixed with saltwater and ecosystem changes occur gradually, creating a land-locked marine lake. The marine lake Kakaban is a highly isolated lake with significantly different ecological, such as salinity, pH, degree of exposure to waves and air [13] compared to the surrounding coastal habitats that possibly force that structure composition of the lake diversity [14]. Hence in such an environment, land-locked marine lake Kakaban provides a unique opportunity to search for novel enzymes, one of which is  $\alpha$ -amylase.

In this study, we report on cloning, expression, and characterizations of an RSDA designated as BmaN2 from a sea anemone associated bacterium *B. megaterium* NL3, isolated from land-locked marine lake Kakaban, East Kalimantan, Indonesia. We believe that none of the previous studies describe raw starch degradation properties of GH13\_36  $\alpha$ -amylase.

## 2. Materials and methods

### 2.1. Materials

All chemicals and solvents used were of analytical grade and were obtained from commercially available sources, i.e., Sigma and Merck (USA). Wizard Genomic DNA Purification (Promega, USA) and Xprep Plasmid DNA Mini Kit (Philkorea Tech, Korea) were used for the extraction of chromosomal and plasmid DNAs, respectively. To purify polymerase chain reaction (PCR) products, GFX™ PCR and Gel Band Purification Kit were purchased from GE Healthcare Life Sciences (USA). Several enzymes used in this study were Dream Taq polymerase (Thermo Fisher, USA), restriction endonucleases *Bam*HI and *Eag*I (NEB, USA), and T4 DNA ligase (Thermo Fisher, USA). For electrophoresis markers, 1 kb DNA ladder was obtained from Thermo Fisher (USA) and ExcelBand™ 3-color Broad Range Protein Marker from SMBIO (Taiwan). Primers used for PCR were synthesized at Macrogen Inc. (Korea). Nickel-NTA agarose (Qiagen, The Netherlands) was applied for BmaN2 purification, and proteins concentration and electrophoresis were assayed with a solution kit from Bio-Rad (USA). Raw starches (wheat, sago, corn, potato, rice, and cassava) were obtained from local supermarkets in Bandung, Indonesia, and canna powder was prepared according to the previous report [15].

### 2.2. Bacterial strains and plasmids

Isolation of bacterium associated with sea anemone from land-locked marine lake Kakaban was carried out in the Center for Tropical Coastal and Marine Studies, Diponegoro University. A colony of sea anemone was collected from a land-locked marine Lake Kakaban, Kakaban Island, part of the Derawan Islands, East Kalimantan, Indonesia, by snorkeling depth of approximately 2 m. Upon collection, the colony was put into sterile plastic bags (Whirl-Pak, Nasco, USA) and stored in a cool-box. The sea anemone tissues were then rinsed with sterile seawater and homogenized. The resultant tissues were serially diluted, spread on ½ strength ZoBell 2216E marine for medium, and incubated at room temperature for 48 h. Based on morphological features, colonies were randomly picked and purified by making streak plates.

For bacterial identification, the 16S rDNA sequence was amplified from chromosomal DNA. The obtained sequences were aligned to other

16S rDNA sequences in NCBI GenBank using BLAST resulted in *Bacillus megaterium* NL3 [16].

*E. coli* TOP10F' (Thermo Fisher, USA) and *E. coli* ArcticExpress (DE3) (Stratagene, USA) were used as a host for cloning and gene expression, respectively. Plasmids pGEMT (Promega, USA) and pET-30a(+) (Novagen, USA) were used for *bmaN2* gene cloning and the gene expression, respectively. Cells were grown in Luria-Bertani (LB) medium (1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract) supplemented with 100  $\mu$ g/mL ampicillin and 30  $\mu$ g/mL kanamycin for selection of pGEMT and pET-30a(+), respectively. Solid media were made by adding 1.5% (w/v) agar to the LB medium. All bacteria were grown aerobically at 37 °C with shaking at 150 rpm.

### 2.3. Isolation of the *bmaN2* gene

The  $\alpha$ -amylase gene from *B. megaterium* NL3 was isolated by the PCR method using a set of degenerate primers, *bma2*-F 5'-ATGAAAG-GRAAAAAATGGACAGC-3' and *bma2*-R 5'-TTATTTTTRTYAAAAAYCGTSGYATTMGC-3'. Both primers were designed based on amino acid sequences of  $\alpha$ -amylase from *B. megaterium* DSM319 (ADF39316.1) and *B. megaterium* QM B1551 (FJ640086.1). The chromosomal DNA of *B. megaterium* NL3 was used as a template for PCR using *Dream Taq* polymerase. PCR was started by an initial denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 95 °C for 1 min, annealing at 46.5 °C for 1 min, polymerization at 72 °C for 1.5 min, and a final extension at 72 °C for 5 min. The amplified DNA was ligated into pGEMT resulted in recombinant plasmid pGbmaN2. The resulted pGbmaN2 were confirmed by nucleotide sequencing (Macrogen, Seoul, South Korea). The sequences of the *bmaN2* gene had been deposited in the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>) under accession number MF521603.1.

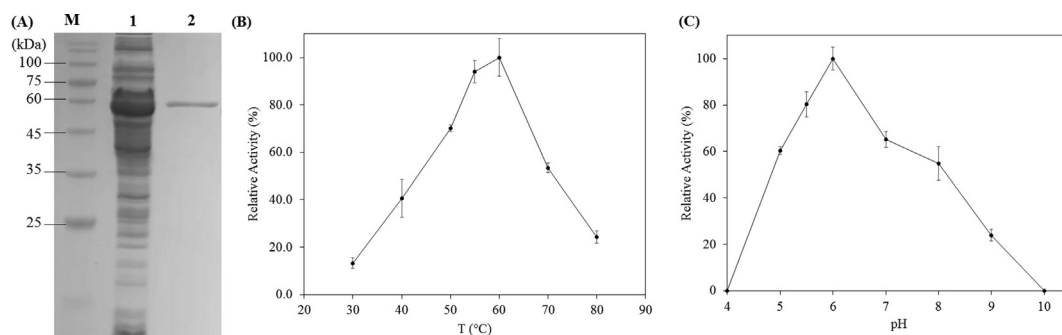
Restriction enzyme sites of *Bam*HI and *Eag*I were introduced to *bmaN2* by PCR using primers, pET-bman2-F 5'-GGATCCGAAACTGTACATAAAGGTAAAGC-3' and pMM-bman2-R 5'-CGGCCGGTGGTGATGGTGATGATGTTATTTTGTAAAACCGTGG-3', and pGbmaN2 as a template. The amplified DNA was ligated into pGEMT producing pGREbmaN2. A *bmaN2* fragment from double digested *Bam*HI-*Eag*I pGREbmaN2 was ligated with a linearized *Bam*HI-*Eag*I pET30a(+) to generate a recombinant pETbmaN2. Subsequently, the recombinant plasmid pETbmaN2 was verified by restriction analysis and DNA sequence analysis.

### 2.4. Expression and purification of recombinant BmaN2

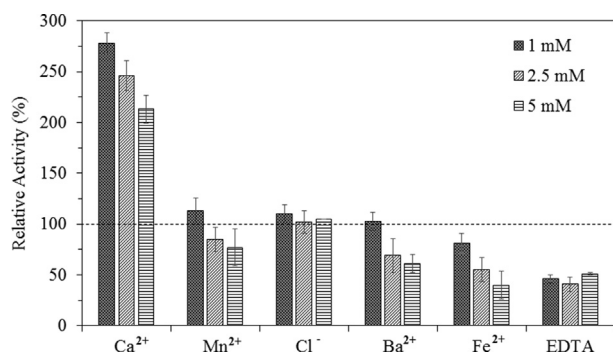
The transformed *E. coli* ArcticExpress (DE3) cells containing pETbmaN2 were cultured in LB medium supplemented with 30  $\mu$ g/mL kanamycin at 37 °C. The overnight culture was transferred into the fresh LB medium and incubated until the optical density of the bacterial culture at 600 nm reached 0.6. Protein production was then induced by the addition of isopropyl- $\beta$ -D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, and the mixture was incubated at 10 °C with shaking at 150 rpm for further 24 h. After centrifugation at 8000 $\times$ g for 20 min at 4 °C, the cell pellet was resuspended in binding buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, pH 8.0) and disrupted by sonication for 15 min with a pulse and an interval time of 30 s. The debris cells were precipitated by centrifugation at 12,000 $\times$ g for 30 min and the crude protein extract was subjected to purification.

The recombinant BmaN2 was purified by the Ni-NTA agarose column chromatography. The Ni-NTA resin was first washed ten times of resin volumes (CVs) by demineralized water and followed by the binding buffer. The crude protein extract was mixed with the resin slurry. Afterward, the mixture was gently shaken at 4 °C for 4 h prior to pouring into the column. Unbound materials were collected as flow-through, and then the resin was washed with four CVs of buffer A (50 mM NaH<sub>2</sub>PO<sub>4</sub> pH 8.0, 300 mM NaCl) with 20 mM imidazole. The bound BmaN2 was eluted from Ni-NTA agarose using three CVs of





**Figure 2.** (A) An SDS-PAGE analysis of BmaN2. Lane M, protein molecular weight marker (kDa); lane 1, cellular proteins from crude extract (pET-30a(+)-bmaN2); lane 2, purified BmaN2 after the Ni-NTA column chromatography. (B) A profile of hydrolytic activity of BmaN2 toward soluble starch in different temperatures and (C) pHs. Optimum temperature and pH of BmaN2 is 60 °C and pH 6.0, respectively.



**Figure 3.** Influence of metal ions and EDTA on the activity of BmaN2 at 60 °C and pH 6.0.

## 2.6. Effect of pH, temperature, and metal ion on the activity of BmaN2

To analyze the effect of pH on the BmaN2 activity, 50 mM universal buffer at pH values ranging from 4.0 to 10.0 was used. The assay mixtures were incubated at 60 °C for 10 min following the DNS method. The optimum temperature was determined over the range of 30–80 °C using the same procedure for determining the optimum pH. The influence of various metal ions on BmaN2 was also investigated. The enzyme was assayed with solutions of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{BaCl}_2$ ,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , respectively, at various concentrations of 1.0, 2.5, and 5.0 mM. Thus the BmaN2 activity in a particular metal ion solution was calculated relatively to the BmaN2 activity without metal ions (100%). The effect of EDTA on the BmaN2 activity was also examined following the same procedure.

## 2.7. Determination of kinetic parameters of BmaN2

The kinetic parameters of BmaN2 were investigated using various concentrations of soluble starch (2.0–20  $\text{mg mL}^{-1}$ ) in 50 mM universal buffer pH 6.0 for 10 min at 60 °C. The amount of BmaN2 used was 0.01 mg. Values of  $V_{\text{max}}$ ,  $k_{\text{cat}}$ ,  $K_{\text{M}}$ , and  $k_{\text{cat}}/K_{\text{M}}$  were obtained from the fitting of the initial reaction rates against concentrations of soluble starch, following the Michaelis-Menten equation. The calculation was performed using GraphPad Prism 7 [18].

## 2.8. Substrate specificity and raw starch digesting analysis

Different types of soluble carbohydrates (soluble starch, glycogen, pullulan, and  $\beta$ -cyclodextrin) were used to determine the substrate specificity of BmaN2. BmaN2 was mixed with 1% (w/v) of each substrate in 50 mM universal buffer at optimum conditions. The enzyme activity with 1% (w/v) of soluble starch was used as a reference (100%).

The ability of BmaN2 in hydrolyzing raw starches was determined using granules from wheat, corn, cassava, potato, rice, sago, and canna.

The mixture, consisted of each raw starch 1% (w/v) and BmaN2 (0.01 mg), was incubated for 48 h with constant shaking (150 rpm) at 37 °C and 60 °C. Subsequently, the mixture was centrifuged, and the amount of reducing sugar, which was released during the enzymatic activity, was measured using the DNS method. The degree of hydrolysis (DH) was defined by the following equation [19]:

$$\text{DH}(\%) = \frac{H_1}{H_0} \times 100$$

where  $H_1$  was reducing sugar produced by enzymatic hydrolysis and  $H_0$  was reducing sugar produced by acidic hydrolysis.

## 2.9. Bioinformatic analysis

The DNA sequences of *bmaN2* were used to perform a homology search using BLASTX (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) [20] against the GenBank non-redundant database. The position of signal peptide cleavage sites in deduced amino acid sequences of BmaN2 was analyzed using SignalP version 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>) [21].

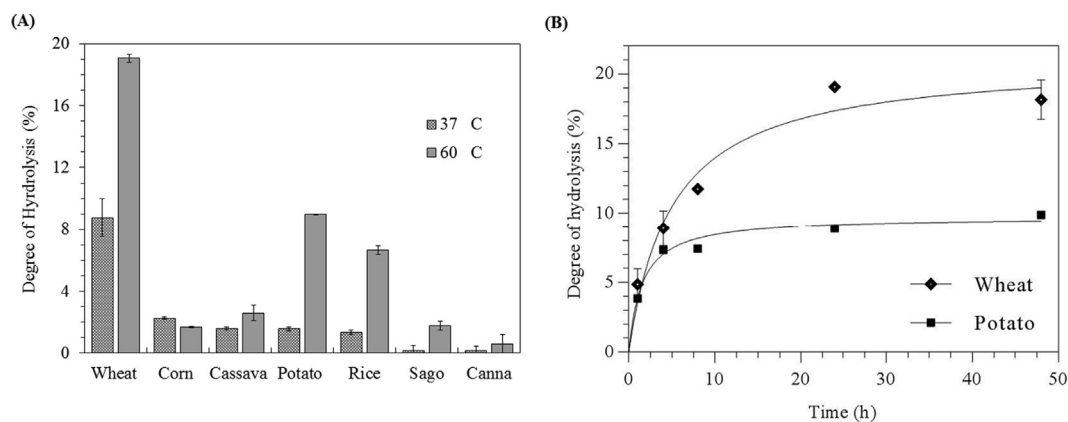
For generating a phylogenetic tree of BmaN2, amino acid sequences of  $\alpha$ -amylases from various subfamilies of GH13 were collected through the CAZy database ([www.cazy.org](http://www.cazy.org)) [3] and GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>) [22]. Multiple sequence alignments of BmaN2 with other  $\alpha$ -amylases from all GH13 subfamilies were performed with the ClustalX 2.1 [23]. A phylogenetic tree was constructed using the neighbor-joining method with a bootstrap value implemented in ClustalX package. The phylogenetic tree was displayed with Figtree version 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>).

The three-dimensional structure of BmaN2 was modeled by I-TASSER (Iterative Threading ASSEMBLY Refinement) server (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>) [24]. As the I-TASSER template, a crystal structure of  $\alpha$ -amylase from *Halothermothrix orenii* (PDB code of 1WZA) was used [25]. Prediction of ligand binding sites was analyzed by MetaPocket 2.0 (<http://projects.biotec.tu-dresden.de/metapocket/index.php>) [26] and analysis of carbohydrate-binding residue was done by SPRINT-CBH (<http://sparks-lab.org/server/SPRINT-CBH/>) [27]. Conserved residues among 198 enzymes of GH13\_36 was analyzed using the ConSurf Server (<http://consurf.tau.ac.il/2016/>) [28]. Visualization of the three-dimensional structure was performed by PyMOL Molecular Graphics System (<https://pymol.org/>) [29].

## 3. Results and discussion

### 3.1. Analysis of nucleotide and amino acid sequences of BmaN2

Recent advances in the genome database mining of microorganisms open an opportunity to explore new enzymes [30]. Based on genomes of *B. megaterium* DSM319 and *B. megaterium* QM B1551, a gene encoding



**Figure 4.** (A) Comparative analysis of the raw starches hydrolysis by BmaN2 at two different temperatures, 37 °C and 60 °C. (B) Hydrolysis of 1% raw wheat and potato starch by BmaN2. Incubation was carried out at pH 6.0 and 60 °C.

extracellular  $\alpha$ -amylase from *B. megaterium* NL3 (*bmaN2*) was PCR amplified. The *bmaN2* ORF is consisted of 1,563 bp encoding  $\alpha$ -amylase BmaN2 with 520 amino acids having the first 25 N-terminal amino acid residues are predicted as a signal peptide.

Homology search using BLASTX against the GenBank non-redundant database [20] revealed that BmaN2 shares 99% identity with putative  $\alpha$ -amylases from *Bacillus sp.*, *Bacillus flexus*, and *Bacillus aryabhatai*, and 97% with  $\alpha$ -amylase from *B. megaterium* WHO, BMW-amy [31]. Moreover, seven conserved sequence regions (CSR) of  $\alpha$ -amylases were also identified in BmaN2 (Figure 1). Three invariant catalytic residues of GH13  $\alpha$ -amylases were also found in the CSR II (aspartate 233), CSR III (glutamate 273) and CSR IV (aspartate 340) of BmaN2 (Figure 1). These results suggest that BmaN2 has the  $\alpha$ -retaining mechanism in hydrolyzing starch as adopted mostly by GH13  $\alpha$ -amylases [32].

BmaN2 was clustered into subfamily 36 of GH13 (Figure 1). The GH13\_36 is defined as a group of amyolytic enzymes which has an intermediate position in the evolutionary tree between the polyspecific subfamilies of oligo-1,6-glucosidase and neopullulanase having a selection marker in the CSR V, MPDLN (Figure 1) [33, 34]. Based on the CAZY database [3], GH13\_36 consists of 381 enzymes from bacteria and eight enzymes from an uncultured bacterium in which only eighteen enzymes have been characterized.

Some of the  $\alpha$ -amylases of GH13\_36 have unique properties, such as AmyA from a thermo-alkaliphilic organism *Anaerobranca gotschalkii* with  $\beta$ -cyclodextrin-forming activity [35]. AmyA from *H. orenii* exhibits high stability in the salt environment, up to 25% (w/v) NaCl [36].  $\alpha$ -Amylase from *Thermotoga maritima* MSB8 has high specific activity [37] and *Xanthomonas campestris* K-11151  $\alpha$ -amylase has broad-specific substrates [38]. Despite their unique properties, there is yet no detailed characterization regarding a raw starch degrading  $\alpha$ -amylase (RSDA) from GH13\_36.

**Table 1.** Gelatinization temperature of raw starches.

Starch source	Gelatinization temperature	References
Canna	68–70 °C	[53]
Cassava	62–72 °C	[54]
Corn	64–73 °C	[54, 55, 56]
Potato	52–72 °C	[54, 57]
Rice	55–79 °C	[55]
Sago	64–80 °C	[54, 58]
Wheat	51–60 °C	[55]

### 3.2. Biochemical characterizations of BmaN2

The purified recombinant BmaN2 was obtained with 5 fold purification, 60% recovery of total activity, and showed a band with a molecular weight of ~62 kDa (Figure 2A). The specific activity of BmaN2 towards soluble starch was  $28.7 \pm 1.4$  U  $\text{mg}^{-1}$ . BmaN2 exhibited activity in a broad pH interval of 5.0–9.0 and a temperature range of 30–80 °C (Figure 2B, C). The optimum activity of BmaN2 was observed at pH 6.0 and 60 °C. These optimum pH and temperature of BmaN2 are similar to that of  $\alpha$ -amylase from *B. subtilis* 65 [39], *B. subtilis* S8-18 [7], and *B. flavothermus* [40].

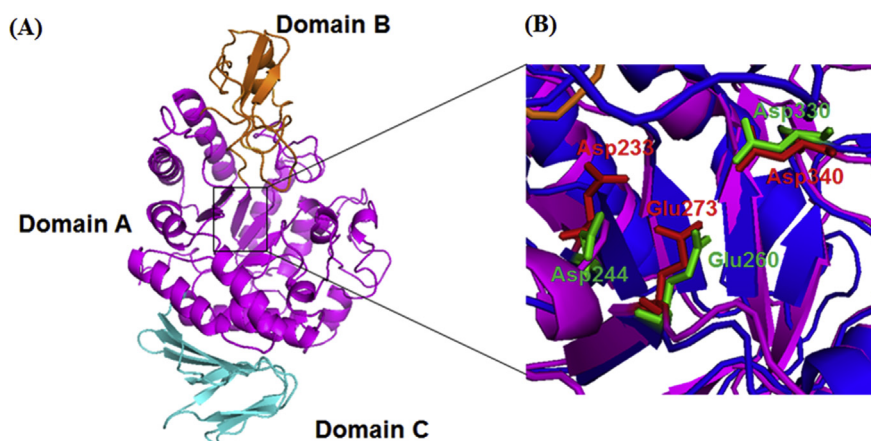
The substrate specificity of BmaN2 was examined toward various soluble substrates, such as soluble starch, glycogen, pullulan, and  $\beta$ -cyclodextrin. BmaN2 activity towards glycogen and pullulan, respectively were 42% and 4%, while no hydrolytic activity was detected on  $\beta$ -cyclodextrin. These results suggest that BmaN2 prefers linear polysaccharide as a substrate, while it is also able to hydrolyze branched substrates at a lower rate.

BmaN2 follows the Michaelis-Menten kinetics profile with soluble starch as the substrate (data not shown).  $K_M$  and  $V_{max}$  values of BmaN2 were  $5.1 \pm 0.9$   $\text{mg mL}^{-1}$  and  $141.6 \pm 8.1$   $\mu\text{g min}^{-1}$ , respectively. Although BMW-amy has smaller  $K_M$  ( $2 \text{ mg mL}^{-1}$ ) than that of BmaN2, the  $k_{cat}$  value of BmaN2 ( $71.5 \text{ s}^{-1}$ ) is three-fold higher than that of BMW-amy [31]. Thus, BmaN2 has catalytic efficiency ( $k_{cat}/K_M$ ) of  $14.1 \text{ s}^{-1}\text{mg}^{-1}\text{mL}$ . The features allow BmaN2 to fit industrial enzyme requirement because BmaN2 requires a large amount of substrate for allowing its maximal activity.

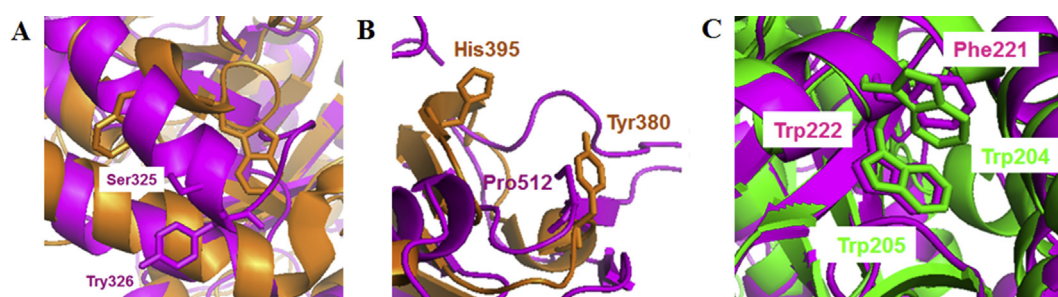
The influence of metal ions on the BmaN2 activity was examined with soluble starch as the substrate (Figure 3). The addition of 1 mM  $\text{Ca}^{2+}$  enhances the activity of BmaN2 significantly to 278%. The presence of  $\text{Ca}^{2+}$  ions up to 5 mM still increases the BmaN2 activity 2-fold. BmaN2 remains unaffected by  $\text{Mn}^{2+}$  and  $\text{Ba}^{2+}$  ions at 1 mM. Conversely, 2.5 mM and 5 mM of  $\text{Mn}^{2+}$ ,  $\text{Ba}^{2+}$ , and  $\text{Fe}^{2+}$  ions have inhibitory effects (Figure 3). The addition of EDTA, a metal-chelator agent, inhibits the BmaN2 leading to 41% remaining activity (Figure 3). These results suggest BmaN2 as a metal-dependent  $\alpha$ -amylase with calcium ion as a positive activator.

#### 3.2.1. Hydrolysis of raw starches by BmaN2

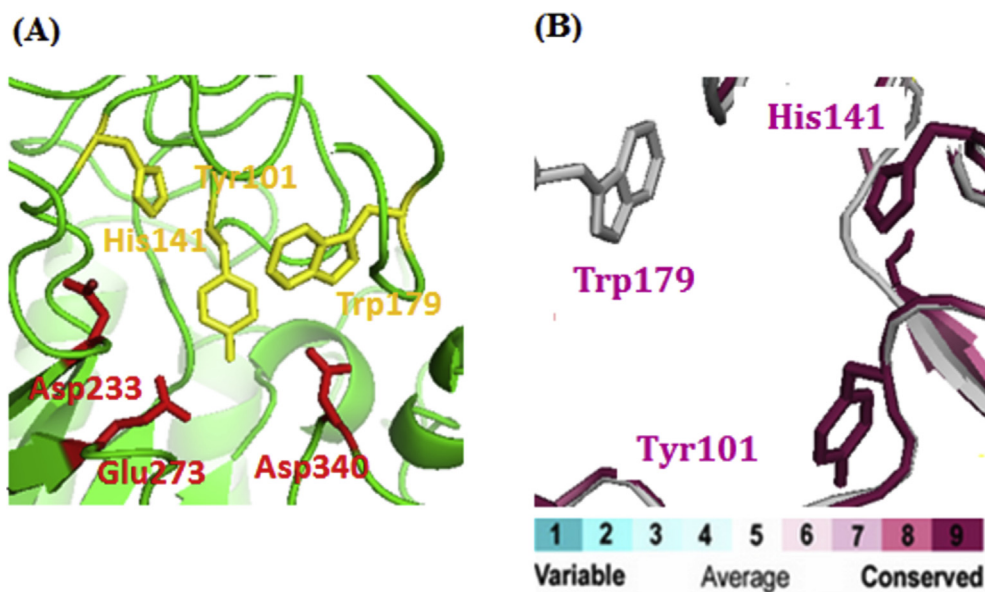
The ability of BmaN2 to hydrolyze various raw starches, corn, rice, canna, cassava, wheat, potato, and sago, was tested at two different temperatures, 37 °C and its optimum temperature 60 °C for 24 h (Figure 4A). Wheat starch is hydrolyzed efficiently by BmaN2, at both temperatures, with the degree of hydrolysis (DH) of 8.8% at 37 °C and 19.1% at 60 °C. Corn, cassava, potato, and rice starches show nearly similar DH of 2.3%, 1.6%, 1.6%, and 1.3%, at 37 °C, respectively. However, potato and rice starches are hydrolyzed preferably by BmaN2 at 60 °C, resulting in DH of 9.0% and 6.7%, respectively.



**Figure 5.** (A) Overall structure the BmaN2 model based on the structure of  $\alpha$ -amylase from *H. orenii*, AmyA (PDB code 1WZA). (B) Superimpose of the conserved catalytic residues of BmaN2 (red) and that of AmyA (green).



**Figure 6.** Superimpose of BmaN2 (magenta) and the structure of AMY1, barley  $\alpha$ -amylase (orange) (A) Substrate Binding Sites (SBS) 1 and (B) SBS 2. (C) Alignment of the structure of GTA (green) and the model of BmaN2 (magenta) in the area surrounding conserved double tryptophan residues.



**Figure 7.** (A) Predicted aromatic residues based on SPRINT-CBH that could be involved in the carbohydrate-binding of BmaN2, Tyr101, His141, and Trp179. (B) Analysis of conserved residues of BmaN2 among the subfamily GH13\_36. Tyr 101 and His141 are highly conserved residues and Trp179 is less conserved in the subfamily GH13\_36.

The starch susceptibility to an enzyme is dependent on many parameters, including gelatinization temperature, where the starch granules begin to swell, absorb the liquid, and result in more reactive to enzyme attack. Wheat and rice starches were found to have low gelatinization temperature, 51–60 °C and 55–68 °C, respectively, compared to other cereal starches (Table 1). Several tuber starches have higher

gelatinization temperature than cereal starches. As shown in Table 1, gelatinization temperatures of canna, cassava, corn, and sago starch were higher than potato starch, which range from 52–72 °C. From this fact, it can be seen that potato, rice, and wheat starches are less resistant to BmaN2 due to the treatment at 60 °C, the temperature at which its starch granules start to lose the crystallinity and amylose solubilized.

BmaN2 exhibits similar optimum temperature and pH to the  $\alpha$ -amylase from *Bacillus subtilis* S8-18. Both enzymes are also able to degrade wheat and potato starches. However, DHs of BmaN2 in hydrolyzing raw wheat and potato are significantly higher than that of amylase from *Bacillus subtilis* S8-18, which is 2.5% and 7.5% for the respective wheat and potato [7]. AmyP,  $\alpha$ -amylase from an unknown marine bacterium, also has small DH values (2,7%) on wheat starch [9] while  $\alpha$ -amylase from *Amphibacillus* sp. NM-Ra2 is able to hydrolyze raw wheat, corn, and potato starch at 50 °C with DH of 14.6%, 13.5%, and 11.3%, respectively [41].

Time-course incubation of raw wheat and potato starches with BmaN2 for 48 h is displayed in Figure 4B. Along with the increase of incubation time, DH of raw wheat arises significantly up to 24 h and BmaN2 is still active towards raw wheat after 48 h. Meanwhile, DH of raw potato escalates only for 4 h to 7.4% and afterwards increase slowly. Different granular structure and crystalline types of raw wheat and potato starches could affect the rate of hydrolysis and BmaN2 substrate preference. Natural starches hydrolysis occurs at a fast initial rate and continues at a slower and more constant rate [42]. Several raw starches hydrolyzed by amylolytic enzymes showed the same pattern curve, such as hydrolysis of potato and wheat of  $\alpha$ -amylase from *Bacillus* sp. IMD 434 [43], glucoamylase from *Corticium roffsii* [44], and rice hydrolysis by AmyASS,  $\alpha$ -amylase from *Aeromonas salmonicida* ssp. *salmonicida* [12].

The different types of starch influence the efficiency of raw starch hydrolysis [45]. Wheat is a cereal starch whose crystalline structure is A-type, whereas potato is a tuber starch formed B-type structure [46]. Most cereals starches' crystalline organization is A-type, which more readily hydrolyses by enzymes than that of B-type tubers starches [47, 48]. Raw starches of B-type of crystalline structure superimposed with larger blocklets at the peripheral level of starch organization, which describe the resistance of starch granules to hydrolysis [47]. Therefore, BmaN2 acts more efficiently on wheat than that of on potato.

To the extent of our knowledge, none of the raw starch activity of  $\alpha$ -amylases from subfamily GH13\_36 has been reported yet. Taken together, BmaN2 is the first  $\alpha$ -amylase from this subfamily which is able to degrade several types of raw starches and it is a potential enzyme to be applied in the food and pharmaceutical industries.

### 3.3. Three-dimensional structure modeling

The generated three-dimensional structure of BmaN2 resembles the AmyA structure from *H. orenii* (PDB code 1WZA), consisting of three domains, a catalytic ( $\beta/\alpha$ )<sub>8</sub> barrel A domain, a small B domain, and a C-terminal domain (Figure 5A). The overall root mean square deviation (RMSD) of both BmaN2 and AmyA was 1.06 Å, implying a highly similar structure. Figure 5B shows conserved catalytic residues of BmaN2, which are highly similar to that of GH13\_36.

Since the model structure of BmaN2 exhibits no additional domain of SBD, suggesting the possibility of substrate binding site (SBS) involved in the digestion of raw starch. Various roles of SBSs have been investigated in the past few years, i.e. targeting the enzyme toward its substrates, guiding the substrate into the active site, and disrupting the substrate [49]. AMY1,  $\alpha$ -amylase from barley, is a well-known raw starch degrading enzyme with two SBS. The first SBS is located in the catalytic domain (Trp278, Trp279) while the second one is in C domain (Tyr380 and His 395) [50]. Superimpose of the BmaN2 model on that of AMY1 shows no similar alignment of the corresponding aromatic residues (Figure 6A, B).

Furthermore, Puspasari et al. postulate the possibility of a conserved pair of tryptophan residues in BaqA involved in the binding of raw starch. The amino acid alignments of BaqA, BmaN2, and several  $\alpha$ -amylases from GH13\_36 imply the conservation of the representative tryptophan and phenylalanine (Figure 1). However, the three-dimensional structure of GTA,  $\alpha$ -amylase from *Geobacillus thermoleovorans*, shows that the two tryptophan residues are not exposed to the surface [51]. Superimpose of the BmaN model structure with the GTA structure shows similar conformation (Figure 6C). Mok et al. predict that hydrophobic sequences (VYVFLFLFLYLIV) in the C domain of GTA plays a role in the degradation

of raw starch. Later, Mehta et al. show that the activity of Gt-amyII without the C domain decreases significantly [52]. Therefore, it is intriguing to search for new types and positions of SBS candidates on BmaN2.

Analysis of amino acid sequences and the model structure of BmaN2 imply that some residues could be candidates for SBS. Prediction on ligand-binding sites and carbohydrate-binding residues generate three possible sites for SBS of BmaN2, Tyr101, His141, and Trp184 (Figure 7A). These residues are located in the BmaN2 active site and highly conserved in the subfamily GH13\_36 (Figure 7B). Surely further investigation on the involvement of these residues as SBS for the raw starch digestion should be done in future research to support this hypothesis.

## 4. Conclusion

This article is the first report of an RSDA with high catalytic efficiency from GH13\_36  $\alpha$ -amylase and a marine bacterium *B. megaterium* NL3. Moreover, the efficient hydrolysis of various raw starches is the advantage of BmaN2. Further details of BmaN2, such as studying the putative SBS of BmaN2 and then engineering of BmaN2 are clearly required to improve its tenacity on digesting raw starch prior to its application in the starch processing industry.

## Declarations

### Author contribution statement

Sofi Siti Shofiyah: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Dewi Yuliani, Nurul Widya, Fean D. Sarian: Performed the experiments.

Fernita Puspasari: Analyzed and interpreted the data.

Ocky K. Radjasa: Contributed reagents, materials, analysis tools or data.

Ihsanawati: Analyzed and interpreted the data; Wrote the paper.

Dessy Natalia: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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### Data availability statement

Data will be made available on request.

### Declaration of interests statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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