Journal of Bio-Molecule Research and Engineering
Vol. 2 | No. 2 |14 – 25 | 2023
e-ISSN: 2962-343X
http://www.e-journal.unair.ac.id/biome

Pycnoscelus surinamensis Endosymbiont Bacteria: Identification and its Amylase Characteristic

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ARTICLE INFO

Article history
Received 16th Sep 2023
Accepted 20th Nov 2023

Keywords:
amylase
endosymbiont
Pycnoscelus surinamensis
Bacillus subtilis
waste recycling

ABSTRACT

The amylase is an enzyme that widely used in industry. In this research, amylase was isolated from endosymbiont bacteria (EKA 4) of cockroach *Pycnoscelus surinamensis* from household garbage dump Surabaya. This research aims to identify bacteria EKA 4, produce and characterize amylase from it at various temperature, pH and effect of metal ions. Amylase screening assay was done by imoculating the isolate into medium contain 2% of starch then incubated for 24 hours. Detection of amylase was done qualitatively by measured presence of halozone and quantitatively using DNS method. 16S rRNA analysis found that bacteria EKA 4 has similarity to *Bacillus subtilis* with 100% of percent identity. Result showed that optimum activity of amylase from *B. subtilis* were at 40°C, pH 6 and could use ion $CaCl_2$ as a cofactor in a reaction. Thus, utilization of amylase from endosymbiont *P. surinamensis* could be as an alternative source in big industries.

1. Introduction

Enzyme is an industrial product which has considerable economic value in the world market nowadays. The utilization of enzymes in the industry is due to its advances in fermentation technology, genetic engineering and applications of enzymes so that the enzyme industry can proliferate and occupy significant positions in the industrial field [1]. Yet industrial-scale manufacturers in Indonesia still have not existed and nearly 99% of the need for domestic enzymes is met by several foreign suppliers from local producers and directly imported from the user industry [2]. Enzyme as strong biocatalysts are produced by a wide range of microbes

which differ in their microbial origins, chemical characteristics, and procesess [3]. Due to their high yields, consistency, ease of product modification and optimization, economic viability, lack of seasonal fluctuations, quick growth of microbes on low-cost media, faster production time on a larger scale, more consistent quality, stability, and elevated catalytic activity, microbial enzymes are preferred [4], [5].

Amylase is one of the hydrolytes produced by microorganisms with widespread distribution and scale [6]. The amylases are distinguished into four kinds based on the catalytic mechanisms, endoamylase, exo-amylase, debranching mechanisms and transferase [7], [8]. Each enzyme has a different catalytic activity and is affected by temperature, pH, substrate and inhibitors, a medium's culture method, medium composition, nutritional needs, incubation times, metal ions and thermal stability and cell growth [9]. Other characteristics of enzymes are the arrangement of amino acids and the molecular weight.

Endosymbionts are organisms which establish a symbiotic association with another cell or creature. Some endosymbionts are intracellular meaning they live inside cells, while others are extracellular meaning they adhere to the cell surface. Relationship that benefit both parties are said to be symbiotic [10]. The bacteria that exist in animal cells can be categorized into a number of groups. The most notable are "primary" symbionts, which live inside specialized host cells called bacteriocytes and have relationships with their hosts that are mutually beneficial and frequently reciprocally obligate. "Secondary" symbionts and intracellular pathogens are more sporadically associated with host individuals and vary in the tissues they occupy. Since the effects on hosts are typically unknown, it is hard to distinguish between symbionts and pathogens; there are undoubtedly a variety of interactions between non-bacteriocyte associates and their hosts [11]. The endosymbiotic microbes support the survival of their hosts and are frequently passed on to the following generation of arthropods by contaminating their eggs [12].

Cockroaches belong to a group of declassified animals that could coexist with a variety of bacteria, thus the bacteria can easily adapt to the cockroaches to form symbiosis [13]. Cockroaches are omnivorous creatures which eat without sifting through trash or waste. A migratory migration in midgut provides a production and secretion of protease enzymes, lipase and amylase. The cockroaches used in this study came from household code garbage in Surabaya. Research conducted by Ni'matuzahroh in 2020 has identified the type of bug as a *Pycnoscelus surinamensis*. As for the isolated bacteria from midgut *P.surinamensis* and used in this study is EKA 4 which has the highest enzyme activity of amylase in early activity testing [14]. Therefore, further research is needed to characterize the amylase of bacteria EKA 4 at various temperature, pH and metal ions to identify the potential applications in industrial field.

2. Materials and methods

2.1. Macroscopic and microscopic identification

Bacteria EKA 4 was inoculated in nutrient agar then incubated for long 24 hours at 37℃. After that, the single colony were observed for their morphological characteristics and stained using Gram staining to identify its microscopic characteristics.

2.2. Molecular identification

The genomic DNA of bacteria EKA 4 was extracted using Wizard Genomic DNA Purification Kit (PROMEGA). Then amplified for the 16S rRNA gene by PCR method using forward primer 27F (5`-AGAGTTTGATCCTGGTCCAG-3`) and reverse primer 1492R (5`-GGTTACCTTGTTACGACTT-3`). The PCR result of the 16S rRNA gene of bacteria EKA 4 isolate was sent to 1st Base for sequence reading. The sequences were aligned using BLAST (Basic Local Alignment Search Tool) which is connected to the NCBI (National Center for Biotechnology Information) server.

2.3. Production of Amylase

Inoculum was made using Nutrient Broth (NB) as a growth medium. Whereas, enzyme production was carried out using media with the following composition: 5 g/L yeast extract, 2.5 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4.7H_2O$, 3 g/L KH_2PO_4, dan 0.25 g/L $CaCl.2H_2O$ into 1000 ml of distilled water. Then added 2% (w/v) of starch as a substrate to the enzyme production medium [15].

Enzyme production was performed by adding 2% (w/v) of culture into 245 ml of production medium and incubating at 30 ℃, speed of 150 rpm, for 48 hours. The culture was then centrifuged at 3500 rpm, temperature 4 ℃, for 30 minutes to obtain cell free supernatant or can be called as crude enzyme. The activity of the amylase was detected using the DNS method by measuring the absorbance of the crude enzyme at λ = 540 nm [16]. The resulting protein estimation was measured using the Lowry method by measuring the absorbance at λ = 750 nm.

2.4. Amylase Activity Assay

As much 0.25 ml of crude enzyme was added to 0.25 ml of substrate (2% w/v dissolved starch in 0.2 M phosphate buffer pH 6) and incubated at 40℃ for 20 minutes. The solution reaction was stopped by adding 0.5 ml of DNS reagent and then homogenized with a vortex. The test tube containing the solution was covered with cling wrap and heated in boiling water for 20 minutes. The solution was cooled and the absorbance was measured at λ=540 nm. Centrifuged starch was used as blank. Enzyme activity was performed using DNS method by determining the amount of reducing sugar formed. The specific activity of the amylase is known through the conversion of the absorbance value of the concentration of the standard glucose curve which is calculated using following formula:

$$AE = \frac{C}{BM\ x\ t} x \frac{H}{E}$$

Note:

AE = enzyme activity (U/mL)
C = glucose concentration (linear regression results of glucose standard curve)
BM = glucose molecular weight = 180 g/mol
T = incubation time (minute)

H = total enzyme - substrate volume (mL)
E = volume of added enzyme (mL)

2.5. Protein Level Measurement

Protein level was measured using the Lowry assay by adding 0.1 ml of sample to 0.5 mL of Lowry C solution, then homogenized using vortex and incubated for long 10 minutes at room temperature. The solution then added 0.05 ml of Lowry D, homogenized and incubated at room temperature for long 30 minutes. The solution was measured for its absorbance at λ=750 nm. Lowry C was used as blank.

2.6. Enzyme Characterization

2.6.1. Effects of Temperature

As much 0.25 ml of 2% (w/v) starch was added to 0.25 ml of crude amylase and then incubated for 20 minutes at various temperatures of 30℃, 40℃, 50℃, 60℃, 70℃, 80℃. After that, approximately 0.5 ml of DNS was added to the mixture and homogenized using vortex. The mixture was reheated in boiling water for 20 minutes and then cooled. The absorbance of the mixture was measured using UV–Vis spectrophotometer at λ=540 nm.

2.6.2. Effects of pH

Around 0.25 ml of 2% (w/v) starch was added to 0.25 ml of crude amylase and then incubated at various pH 4, 5, 6, 7, and 8. Conditioning for pH 4 and 5 was used citrate buffer while pH 6, 7, and 8 was used phosphate buffer. Crude amylase activity was measured by adding 0.5 ml of DNS and homogenized with a vortex. Hereafter, the absorbance of the solution was measured at λ = 540 nm.

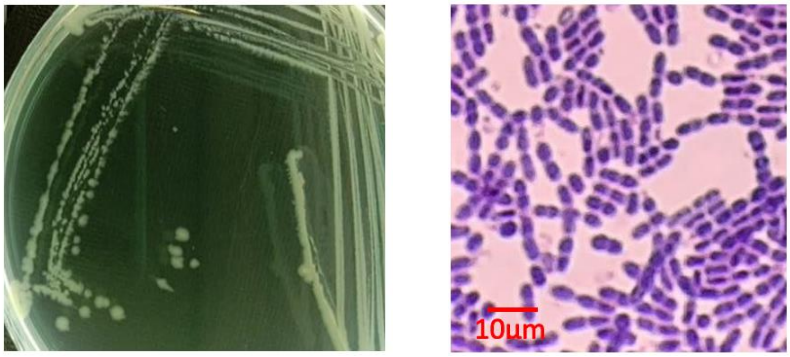
2.6.3. Effects of Metal ions

As much 0.25 ml crude enzyme was added to 0.25 ml starch substrate 2% (w/v) then added with 0.1 ml metal ion and incubated at 40℃ for long 20 minutes. The types of metal ions used in this study consisted of 1 mM $MgSO_4$, $FeCl_3$, $CuSO_4$, $CaCl_2$, and $MnSO_4$. Furthermore, enzyme activity was measured using the DNS method by adding 0.5 ml of DNS reagent and homogenizing. The mixture was then heated in boiling water for 10 minutes and cooled. The absorbance of the mixture was measured using a UV–Vis spectrophotometer at λ=540 nm.

3. Result and Discussion

Amylase is known as catalyst for the hydrolysis of starch into oligosaccharides [17]. Commonly, amylase could be found in plants, animals, fungi and bacteria. Nevertheless, amylase produced by bacteria are more widely used due to its stabilization [18]. Amylase have some essential roles in manufacture of detergent, textile, alcohol and pulp. Recently, amylase is being the most utilized enzyme in industry following by its used around 25% from the total used of enzyme in the world [19].

This research used bacteria EKA 4 isolated from endosymbiont of cocroach *Pycnoscelus surinamensis.* The characteristic of this bacteria could be seen on figure 1. down below:



(a) **(b)**

Figure 1. (a) Macroscopic characterization of bacteria EKA 4 shows that its colony has irregular shape in pinpoint size in white, edge of type is entire and raised elevation with smooth and glistening surface, the opacity is opaque and has vicid consistency; **(b)** Microscopic characterization of bacteria EKA 4 which has rod-shaped cell and belong to the group of Gram positive bacteria that indicated by cell that shown a purple color after Gram staining assay.

Molecular identification was done by isolating the DNA genome of Bacteria EKA 4. The result of DNA isolation was then evaluated for purity and concentration with data such as on table 1. down below:

Table 1. Purity and concentration of Bacteria EKA 4

Isolate code	Absorbance			Concentration (µg/mL)	Purity (λ260/λ280)
	230 nm	260 nm	280 nm		
EKA 4	0,361	0,546	0,304	27,30	1,80

Based on the table 1. above shows that bacteria EKA 4 has a concentration of 27.30 µg/mL with DNA purity of 1.80 (λ260/λ280). DNA purity is measured by comparing absorbance on a wavelength of 260/280 nm. In principle, measuring the number of DNA using a spectrophotometer based on UV-irradiation absorbed by nucleotides and proteins in the solution. DNA absorbs the strongest or maximum exposure to UV radiation at wavelength of 260 nm while protein absorption is achieved at quality length of 280 nm [20]. Good quality of DNA purity is in the range of 1.8 - 2.0. If comparative value of λ260/λ280 shows a purity value less than 1.8 it means each DNA contain of phenol and the remaining solvent of DNA. Whereas, if the purity value shows more than 2.0 it indicates that DNA isolate is still contain contaminants such as proteins and other cellular components or possible cellular lymphatic processes [21].

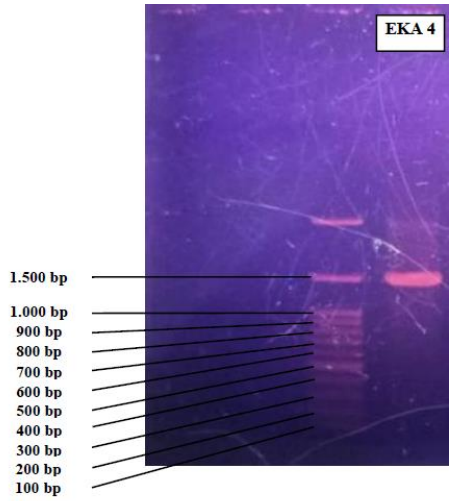


Figure 2. PCR Visualization of Bacteria EKA 4 using 16S rRNA analysis

Electrophoresis gel agarose 1% of bacteria EKA 4 based on figure 2. shows a single piece of DNA seen in the sample column of size over 1500 bp. The results of sequencing DNA in the sequential genome of bacteria EKA 4 were good indicated by a thick, clear and lack of a smear on the DNA tape. The BLATS-N analysis of the bacteria EKA 4 could be seen in table 2. Based on the table suggests that bacteria EKA 4 has similar in nature to *Bacillus subtilis* with a number of percent identity is 100% with query cover value of 98%.

Table 2. BLATS-N result of Bacteria EKA 4

Isolate code	Name of species	Per. identity	Query cover	Acc. Number
EKA 4	*Bacillus subtilis*	100%	98%	NR_118383.1

Genus *Bacillus* belongs to the Firmicutes [22], [23]. Since its role as the second most commonly grown bacterial phylum from cockroaches; The mid gut has the highest concentration of firmicutes (~43%), followed by the fore gut (30%) and the hind gut (34%) [24]. Numerous alkaliphilic, aerobic bacteria belonging to the genera *Bacillus, Paenibacillus,* and *Enterococcus* thrive in the mid gut due to its more alkaline nature (pH 6.1–8.9) [25], [26]. Another study related to Bacillota isolated from the insects gut environment also shown its abilities in some enzymes hydrolitic for instance Amylase, Cellulase, Protease, Lignocellulase, Xylanase, Lipase, Pectinase, Esterase, Chitinase, and Linamerase [27].

Among the microbes that produce amylase, genus *Bacillus* is known as a microbe that commonly used as an amylase producer [19], [28]. Various organic substrates such as glucose, maltose, maltoriose and lactose had been studied for their effectiveness in amylase production. The use of starch as a substrate shows a higher amylase product than other organic substrates [29]. Rathore and Singh (2021) said that starch is the main substrate in amylase fermentation by bacteria. Therefore, starch has been selected as a substrate in this research [30].

Amylase production was done in 24 hours incubation. According to Ni'matuzahroh, et al (2020), 24 hours incubation is the optimum condition to produce amylase from *Bacillus subtilis* (EKA 4) [14]. Amylase detection secreted by *Bacillus subtilis* EKA 4 could be known from qualitative measurement by observing the presence of halo zone on isolate grown on medium containing 2% of starch and quantitative measurement using DNS method. As shown at (figure 3.) halo zone was formed indicate that *Bacillus subtilis* (EKA 4) could produce amylase in size as large 1,675 cm.

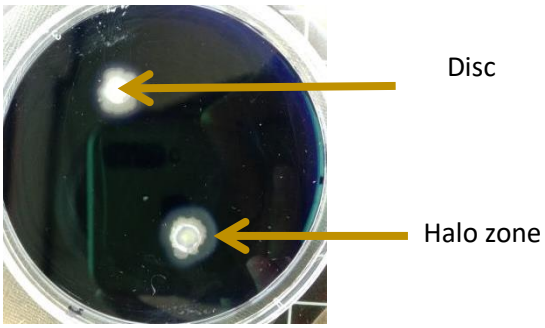


Figure 3. Halo zone presence of bacteria EKA 4

Table 3. Quantitative measurement of Bacteria EKA 4 using DNS Method

	Amylase activity (U/mL)	Protein level (mg/mL)	Specific enzyme activity (U/mg)
Average	0,643 ± 0,057	3868,77 ± 0,012	0,166 ±0,019

Whereas, according to the table 3. above, the average value of amylase activity of bacteria EKA 4 is 0.643 ± 0.057 U/ml. Besides, the average value of protein level is 3868,77 ± 0,012 mg/ml. Moreover, 0,166 ±0,019 U/mg is the average value of specific enzyme activity by *Bacillus subtilis* (EKA 4).

Furthermore, each crude enzyme from *Bacillus subtilis* (EKA 4) was characterized by several factors such as the effect of heating temperature variation, pH and the addition of metal ions. Based on the result, known that amylase produced by *Bacillus subtilis* (EKA 4) has an optimum activity at 40℃ with the amount of amylase activity is 0.627 U/mL (Figure 4). Amylase produced by *Bacilus* is optimum at 40℃ [31]. It also has ability to growth properly at pH 6 with a value of 0.587 U/mL (Figure 5.) [32], [33]. Previous research shown the optimum pH of amylase production by starch is around 6,0 - 7,5 [34], [35], [36], [37]. Moreover, the addition of metal ions as much as 1 mm each ions shows that $CaCl_2$ could be a cofactor to increase enzyme activity seen by its highest value in an assay while other metal ions were act as an inhibitor (Figure 6).

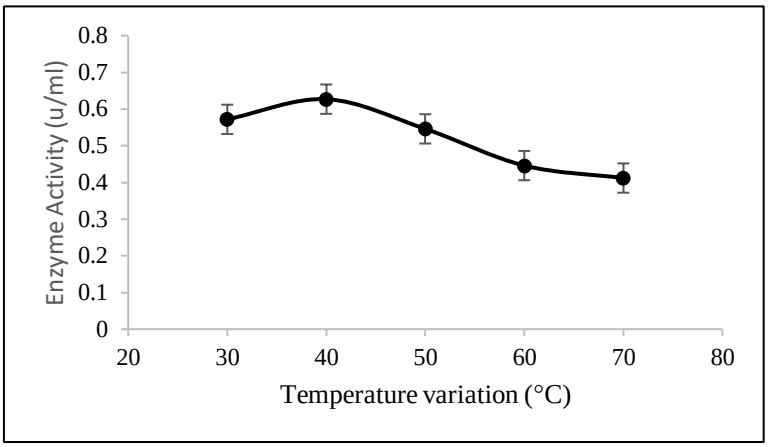


Figure 4. Amylase activity of *Bacillus subtilis* (EKA 4) at various heating temperature (U/mL).

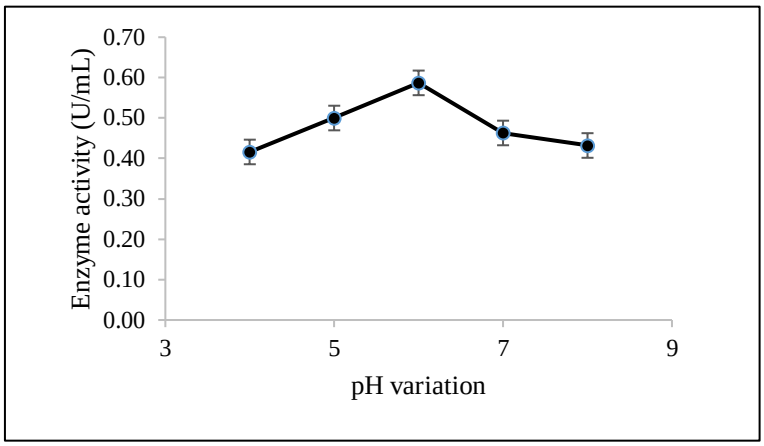


Figure 5. Amylase activity of *Bacillus subtilis* (EKA 4) using variation of pH (U/mL)

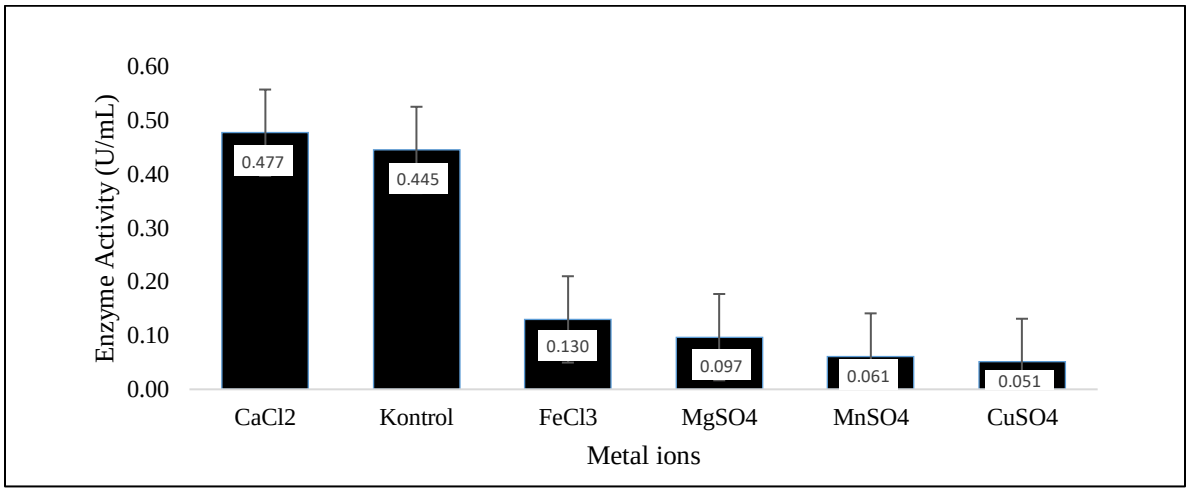


Figure 6. Amylase activity of *Bacillus subtilis* (EKA 4) using addition of metal ions (U/mL)

Several studies have shown that amylase activity in cockroach digestion was influenced by some factors such as type of food and stage of growth (metamorphosis). As explained by Vinokurov *et al* [25] that cockroaches were fed with different food are tend to produce varying amylase activity. The metamorphosis experienced by cockroaches in larva and adult stage are also cause a specific regulation of amylase due to its different food intake that affect the expression of AMY gene as an amylase encoding [38].

Throughout this research could be known that endosymbiont bacteria in cockroach digest is able to produce various hydrolytic enzymes, one of which is amylase. Related to cockroach role in nature, enzyme produce by endosymbiont microbes in its digest would parsing plant biomass, carbon and nitrogen circle also rate of nitrogen fixation [39].

4. Conclusions

The endosymbiotic bacterium *Pycnoscelus surinamensis* was recognized as *Bacillus subtilis* with percent identity as much 100% and query cover as much 98% . It has irregular shape in pinpoint size with white color, type of edge is entire, raised elevation with smooth and glistening surface preference, opaque in opacity and vicid in consistency. Whereas, its microscopic is rod-shaped cell and belong to Gram positive bacteria. This isolate is able to produce amylase, which has highest activity at 40℃ and pH 6, and utilizes $CaCl_2$ as a cofactor in its enzymatic reaction.

Acknowledgements

This research received funding from the Direktorat Riset dan Pengabdian Masyarakat, Deputi Bidang Penguatan Riset dan Pengembangan Kementerian Riset dan Teknologi/ Badan Riset dan Inovasi Nasional through the Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT) scheme, year 2021, under contract number 472/UN3.15/PT/2021. The authors extend their gratitude to the students of the Biology Department at Universitas Airlangga for their cooperation and support.

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