

Activity of Proteolytic and Amylolytic Enzymes from *Bacillus* spp. Isolated from Shrimp Ponds

IT JAMILAH^{1,2}, ANJA MERYANDINI¹, IMAN RUSMANA^{1*},
ANTONIUS SUWANTO¹, AND NISA RACHMANIA MUBARIK¹

¹Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor,
Darmaga Campus, Bogor 16680, Indonesia;

²Department of Biology, Faculty of Mathematics and Natural Sciences,
Universitas Sumatera Utara, Jalan Prof A Sofyan No 3, Medan 20155, Indonesia

Accumulation of feed excess in commercial shrimp ponds due to overfeeding could decrease water quality. Protein and starch are the primary components of shrimp feed. This study was conducted to characterize extracellular proteases and amylases of *Bacillus* spp. isolated from shrimp ponds. 72 proteolytic and amylolytic *Bacillus* spp. isolates were screened from shrimp ponds in Karawang, West Java. Ten isolates were selected for further characterization for their growth and ability to reduce total suspended solid generated from commercial shrimp feed. *Bacillus* sp. DA 5.2.3 and L5 showed excellent activity in reducing total suspended solid, by 37 and 30% respectively. Protease and α -amylase activities of *Bacillus* sp. DA 5.2.3 isolate were consistently higher than that of L5. Maximum total and specific protease activity of DA 5.2.3 isolate was 2.0 U mL⁻¹ and 40.9 U mg⁻¹ respectively, while the activities of the L5 isolate were 2.1 U mL⁻¹ and 23.0 U mg⁻¹ respectively. Based on its 16S rRNA gene sequences, *Bacillus* sp. DA 5.2.3 showed 99% similarity to *Bacillus cereus* XHJ-2-6. *Bacillus* sp. DA 5.2.3 could potentially be applied to maintain water quality by reducing total suspended solid in water columns of shrimp ponds.

Key words: *Bacillus* sp. DA 5.2.3, protease, α -amylase, total suspended solid

Overfeeding in aquaculture system can influence water quality that hampers animal growth. Generally, 10% of feed remains as waste in the water. A high load of feed in pond water can increase biological oxygen demand (BOD), bacterial population, and decrease dissolved oxygen (DO) content. It can also increase total suspended solid (TSS) in the water. Reduction of water quality has a bad influence on survival and growth of shrimps.

The used of bacterial probiotics in shrimp aquacultures has been studied and reported to have great impact on shrimp growth and survival. Some of these can maintain water quality by reducing the ammonia concentration in shrimp ponds, others can inhibit the growth of shrimp pathogens (Rengpipat *et al.* 1998; Vaseeharan and Ramasamy 2003; Decamp and Moriarty 2007), stimulate the immune system of the shrimp (Gullian *et al.* 2004) and increase digestive enzyme activity of the shrimps intestine (Ziaei-Nejad *et al.* 2006).

Bacillus spp. have derived great attention as a probiotic application in aquaculture in the last decade (Rengpipat *et al.* 1998; Vaseeharan and Ramasamy 2003; Decamp and Moriarty 2007). Many researchers reported their roles as biological control agents in shrimp ponds (Verschuere *et al.* 2000; Decamp and Moriarty 2007). Recently, Ochoa-Solano and Olmos-Soto (2006) studied the contribution of *Bacillus* as part of the functional feed to enhance shrimp feed quality. Several studies of potential protease from *Bacillus cereus* isolated from fish gut for converting fish-wastes have been reported. However, information about the role of *Bacillus* spp. to reduce shrimp feed wastes, was very limited. The group of *Bacillus* bacteria is well known as a producer of a large variety of extracellular protease and amylase. Therefore, this study attempted to identify potential proteolytic and amylolytic enzymes of *Bacillus* spp. isolated from shrimp

ponds for their potential application to maintain water quality in shrimp aquaculture.

MATERIALS AND METHODS

Sample Collection and Preparation. Nineteen samples of four different sample sources being soil, sediment and water and shrimp intestine were collected from shrimp ponds in Karawang, West Java, Indonesia. From samples of shrimp intestine were prepared by grinding and diluting in 0.85% (w/v) NaCl.

Isolation and Screening of *Bacillus*. Serial dilution of each sample in 0.85% NaCl (w/v) was follow by heating at 80 °C for 15 min. Then, 100 mL aliquots solution was spread on half strength of solid seawater complete medium (SWC) (Atlas 1997) added with 1% (w/v) skimmed milk. Cultures were incubated at room temperature 24-48 h. Proteolytic activity was shown by formation of clearing zones around colonies. Amylolytic activities of *Bacillus* spp. were screened by a similar method except SWC medium was mixed with 1% (w/v) commercial cassava starch instead of glycerol was used as the carbon source. Clearing zones around the colonies were detected by iodine staining solution (15 g L⁻¹ potassium iodide and 15 g L⁻¹ iodine in distilled water). The proteolytic index (PI) or the amylolytic index (AI) value is the ratio of hydrolysis zone (clear zone) diameter (cm) formed by a bacterium colony and its colony diameter (cm).

Proteolytic and amylolytic activity positive colonies were isolated on solid SWC medium to give a pure culture. Confirmation of amylolytic and proteolytic activity of the pure culture isolates were conducted using similar methods as to mention above.

Growth Conditions of Selected Isolates. Selected high PI and AI value isolates were inoculated into 50 mL SWC medium in 250 mL flasks and then incubated on shaker (120 rpm) at room temperature (28 °C) to get 10⁸ CFU mL⁻¹ (OD_{620nm} = 0.4).

*Corresponding author., Phone/Fax: +62-251-8622833,
E-mail: rusmana13@yahoo.com

Then, 10 mL aliquots of cultures were inoculated into 100 mL of SF medium with 2.5% (w/v) salinity and pH 7.5. Cell densities were determined at 24 and 48 h.

Total Suspended Solid (TSS). Fifty mL of 1.2% (w/v) shrimp feed (SF) medium (pH 7.5) was inoculated with 10 mL of bacteria with a concentration of 10^8 CFU mL⁻¹. Incubation was run at room temperature on a shaker (120 rpm) for 92 h. TSS values were determined using filtration method.

Enzyme Production. Bacteria were grown in 1.2% (w/v) SF medium adjusted to pH of 7.5 and incubated in a shaker at 120 rpm at room temperature for 96 h. Samples were taken at a 24 h time interval then centrifuged at 4500 x g for 15 for min (Jouan C31) at 4 °C. The clear supernatant was used as the crude enzyme preparation.

Protease Activity Assay. The substrate used for protease activity assay was 1% (w/v) casein in 0.01 M Tris-HCl buffer at pH 7.5. Tyrosine 0.37 mmol L⁻¹ was used for a standard. Each mixture enzyme reaction was incubated at room temperature for 10 min and the reaction was stopped by adding of 10% (w/v) trichloro acetic acid (TCA). All tests were done in replicates. Protein concentration was measured using a colorimetric method (Bradford 1976). Bovine serum albumin was used as a standard. One unit of the enzyme activity (1U) is defined as the amount of enzyme needed to produce 1 µmol tyrosine per min, while specific activity of an enzyme is a unit of enzyme activity divided by its protein concentration.

Amylase Activity Assay. Assay of α-amylase activity was measure based on the colorimetric method (Benfeld 1955). Soluble starch at 1% (w/v) was dissolved in 0.05 M Tris-HCl buffer at pH 7.5 and used as a substrate. One unit of enzyme activity (1U) is defined as an activity of the enzyme to produce 1 µmol maltose per min.

Bacillus Identification. Identification of selected isolate was conducted based on morphology characterization. It was then confirmed with 16 S rRNA gene sequencing. DNA extraction was performed based on the CTAB (*cetyl-trimethyl-ammonium bromide*) method. The 16S rRNA gene was amplified with polymerize chain reaction (PCR) machine (Gene Amp PCR system 2400, Perkin Elmer, Biosystem, USA) using a specific primer for bacteria (63f (5'-CAGGCCTAACACATGCAAGTC) and 1387r (5'-GGGCGGWTGTACAAGGC) (Marchesi *et al.* 1998) (Research Biolab, Singapore). DNA was sequenced (Applied Biosystem, USA) and Blast analysis was conducted (NCBI: at <http://www.ncbi.nlm.nih.gov>).

RESULTS

Screening of Bacteria. There were 72 *Bacillus* spp. isolated from 19 samples taken from shrimp ponds in Karawang, West Java. All isolates assayed positive for

proteolytic and/or amylolytic activities, were Gram positive and were rod shaped. Colony morphology of the isolates found generally were white-cream or cream in color, concentric or circle in shapes, entire and wave or ununiform in their margin morphology.

Isolation results showed that sediment was the most prolific source of *Bacillus* sp. compare to other sources, giving 36% followed by soil that gave up to 29% of the total isolates found. The lowest number of *Bacillus* spp. found was in the water samples (15%), however it was higher than that in shrimp intestine samples. Most of the isolates found (90.3%) had both proteolytic and amylolytic activities, which could be seen from clearing zone produced around colonies in half strength SWC-milk and SWC-starch medium (Table 1).

The proteolytic index (PI) and the amylolytic index (AI) of the isolates were varied. The PI value of the isolates isolated from soils were in the range of 1.2 and 6.9 while AI values were in the range of 1.5-5.6. The average of the PI and AI was 3.7 and 3.0 respectively. The L3, L5 and KAt 5.1 isolates had a high value of either proteolytic or amylolytic indexes. These isolates were selected for further study. The PI and AI values of the isolates isolated from pond sediment were in the range of 1.5-7.2 and 1.2-8.3 respectively. The average of PI and AI values were 3.4 and 3.0 respectively. KPt 7.1.1 and KPt 7.1.2b isolates had a high values of either PI or AI. The average of PI and AI values of the isolates isolated from water were 2.6 and 3.6 (Table 1). The isolates isolated from shrimp intestines mostly had higher values of PI and AI than isolates of other sources. The average values of PI and AI of the isolates were 5.8 (in the range of 2.7-18.8) and 5.3 (in the range of 1.4-7.8). The highest values of PI was performed by DA 2.2.1 isolate, but its AI value had a lower value than other isolates. The DA 5.2.3 isolate had high values of both PI and AI. This isolate was selected for further study. The DP 3.2.1 isolate had only proteolytic activity in contrast, others, such as DA 2.2.3, UA 1.2.1, UA 1.2.2 isolates only assayed positive for amylolytic activity.

Ten isolates were selected for further characterization based on their PI and AI values (Table 2). All isolates selected were Gram positive, rod-shaped, catalase positive and endospore-forming bacteria. When the isolates were grown in a solid SF medium, all were able to degrade starch of shrimp feed, denoted by formation of a clear zone. However, the clearing zones did not show proteolytic activity (unpublished data). In general, the AI value of the isolates growing in SWC starch medium were higher than of those grown in SF medium.

Growth of Selected *Bacillus* spp. All selected isolates grew well in SF medium (Fig 1a and 1b). However, they had different growth rates. The bacteria grew fast for the first 24 h. The increase of the cell numbers was as high as $5 \log_{10}$ to $8 \log$ CFU mL⁻¹ and then the growth rate was reduced by further

Table 1 Isolated proteolytic and amylolytic *Bacillus* from shrimp ponds in Karawang, West Java

Sources	Number of		Total of isolate	% of isolate	Ranges of hydrolysis zone		Average of hydrolysis zone			
	Samples	P and A			P	A	PI±SE	AI±SE	PI±SE	AI±SE
Soil	6	18	1	2	21	29	1.2±0.1 to 6.9±2.1	1.5±0.1 to 5.6±1.4	3.7±0.3	3.0±0.3
Sediment	4	26	0	0	26	36	1.5±0.0 to 7.2±0.1	1.2±0.4 to 8.3±1.0	3.4±0.4	3.0±0.3
Water	5	11	0	0	11	15	1.5±0.0 to 3.6±0.4	2.0±0.5 to 4.8±0.2	2.6±0.2	3.6±0.2
Intestine	4	10	3	3	14	19	2.8±0.1 to 18.8±0.3	1.4±0.3 to 7.8±0.3	5.8±0.4	5.3±0.6

P, proteolytic; A, amylolytic; PI, proteolytic index; AI, amylolytic index; SE, standard error.

Table 2 Ten of 72 *Bacillus* spp. isolated from shrimp ponds

Sources	Isolate codes	Hydrolysis index	
		PI±SE	AI±SE
Soil	L3	3.7±0.4	3.5±0.5
	L5	4.7±0.6	3.5±0.3
	KAt 5.1	3.3±0.2	5.2±0.6
Sediment	KPt 7.1.1	7.2±0.1	2.5±0.0
	KPt 7.2.1	6.6±0.9	4.0±1.4
	bKAt 7.1.1	2.3±0.1	8.3±1.0
Water	NS		
Intestine	DA 2.2.1	18.8±0.3	3.6±0.4
	DP 5.1.1	4.2±0.6	6.0±1.6
	DP 5.1.2	4.0±0.3	4.8±1.4
	DA 5.2.3	4.7±0.3	7.8±0.3

PI, proteolytic index; AI, amylolytic index; SE, standard error; NS, not selected.

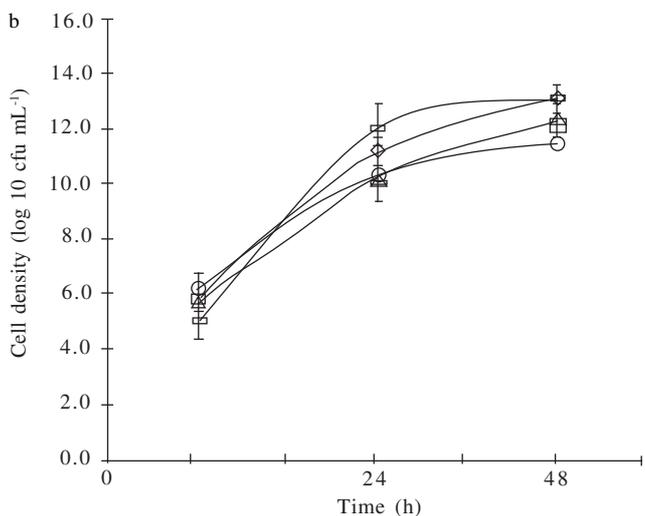
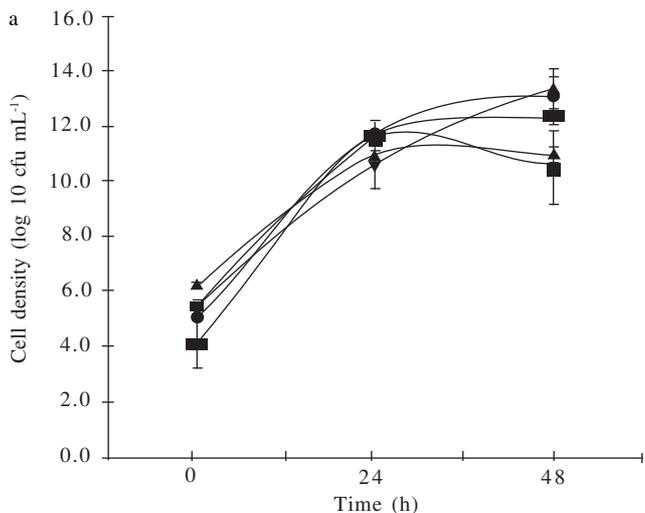


Fig 1 The growth of selected isolates of *Bacillus* spp. as probiotic candidates over incubation time in commercial shrimp feed medium a: ◆, isolate KAt 7.1.2; ■, isolate DP 5.1.1; ▲, isolate DP 5.1.2; ■, isolate DA 2.2.1; ●, isolate DA 5.2.3. b : ◇, isolate L3; □, isolate L5; △, isolate KAt 5.1; □, isolate KPt 7.1.2b; ○, isolate KPt 7.1.1.

incubation up to 48 h. The increase of the cell was as much as 1 to 2 log₁₀ CFU mL⁻¹. The data showed that a logarithmic phase was reached before 24 h of incubation, followed by a stationary phase after 48 h of incubation. The highest growth

rate was performed by L5, DP 5.1.1 and DA 5.2.3 isolates. The number of cells increased up to 7 log₁₀ CFU mL⁻¹.

Total Suspended Solid (TSS). Reduction of TSS was measured using 25 mL 1.2% SF medium in 250 mL flask, incubated on shaker at room temperature (28 °C) for 92 h. The highest reduction of TSS was performed by L5 and DA 5.2.3 isolates, which decreased TSS up to 36 and 30% respectively (Table 3).

Protease Activity. *Bacillus* sp. DA 5.2.3 and L5 were characterized of their protease activities in commercial SF as medium production. The DA 5.2.3 isolate showed higher proteolytic activity than the L5 isolate. The proteolytic activity of *Bacillus* sp. DA 5.2.3 was 2.0 U mL⁻¹ higher than that of *Bacillus* sp. L5 (1.4 U mL⁻¹). However, specific activity graphs of both isolates had a similar pattern. The highest specific activity of DA 5.2.3 and L5 isolates was 40.9 U mg⁻¹ and 23.0 U mg⁻¹ respectively (Fig 2).

Amylase Activity. Total α-amylase activity and its specific activity for DA 5.2.3 were also higher than that of the L5 isolate. The highest total α-amylase activity of the DA 5.2.3 and the L5 isolate was reached in 24 h of incubation, which were 2.1 U mL⁻¹ and 0.5 U mL⁻¹ respectively (Fig 3). The activity of this enzyme subsequently decreased with the time of incubation. Specific activity of the DA 5.2.3 isolate was higher than that of the L5 isolate, which were 47.3 U mg⁻¹ and 9.1 U mg⁻¹ respectively.

Identification of *Bacillus* sp. DA 5.2.3. Morphology characterization of DA 5.2.3 isolate showed that this isolate

Table 3 Total suspended solid (TSS) of *Bacillus* spp. cultured in 1.2% commercial shrimp feed medium

Isolates	TSS (mg mL ⁻¹)	TSS decreasing (%)
L5	1640	36.9
DA 5.2.3	1840	30.4
DP 5.1.1	1880	27.6
KPt 7.1.1	1880	27.6
KAt 5.1	1920	26.0
K.Pt 7.1.2b	2000	23.0
L3	2240	13.8
KAt 7.1.2	2320	10.8
DP 5.1.2	2400	7.7
Control	2600	0.0

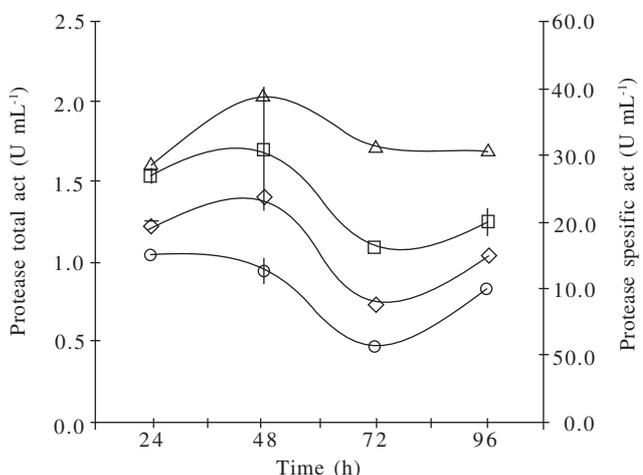


Fig 2 Protease activity of *Bacillus* sp. L5 and DA 5.2.3 over time production. △, DA 5.2.3 total act; □, DA 5.2.3 specific act; ◇, L5 total act; ○, L5 specific act.

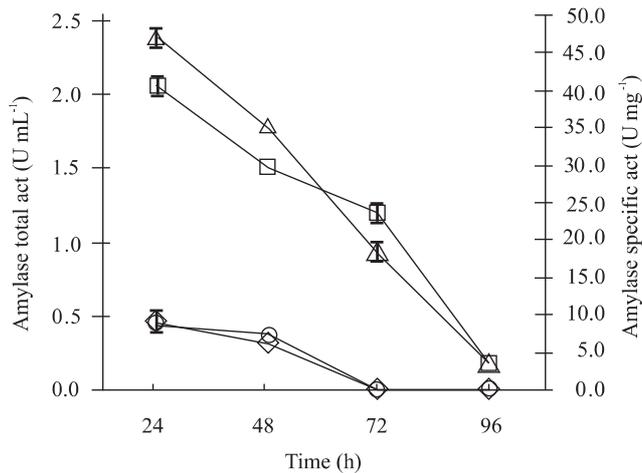


Figure 3 Alpha-amylase activity of *Bacillus* sp. L5 and DA 5.2.3 over time production. □, DA 5.2.3 total act, △, DA 5.2.3 specific act; ○, L5 total act; ◇, L5 specific act. Bars indicate standard error.

was Gram positive, rod in shape, catalase positive and an endospore-forming bacterium. PCR amplification of its 16S rDNA gene showed a 1.3 kb band on electrophoresis. Blast analysis of this gene sequences (NCBI) indicated that this isolate had the highest 16S rDNA gene sequences similarity (99 %) with *Bacillus cereus* XHJ-2-6 (accession number: GQ 19959.1).

DISCUSSION

Higher numbers of *Bacillus* isolated from sediment may be expected because of sediment is at the bottom site of pond, and more nutrients are concentrated there. On the contrary, *Bacillus* isolated from shrimp digestive tract were the lowest in number. A low number bacteria was found in water samples compare to others site of ponds. Bacteria in aqueous environment can reach the animal digestive tract through drinking or swallowing of water by the animals. Lee *et al.* (2000) stated that one of factor for competency of probiotics was their ability to attach to intestine mucus. Therefore, only selected bacteria could survive in the intestine.

More than 90% isolates selected had both proteolytic and amyolytic activity. And most of *Bacillus* spp. isolates have more than one extracelullar enzymes. *Bacillus* spp. isolates taken from the shrimp digestive tract had the highest values of PI and AI. This could be due to a lot of nutrient being concentrated in intestine, so that only bacteria that have potential enzyme activity to degrade substrates could survive and could be more competitive in the intestine. Denkin and Nelson (1999) reported that the production of potease activity in fish digestive tract mucus of *Vibrio anguillarum* was higher than other media.

Selected isolates of *Bacillus* had the same growth pattern, and their logarithmic growth phase was reached at 24 h of incubation. Santos and Martins (2003) reported that logarithmic growth phase of *Bacillus* grown on 1% soluble starch and 1% maltose was reached at between 18 and 48 h of incubation.

The ability of the selected isolates to reduce total suspended solid values has potential application to maintain water quality in terms of total suspended solid reduction. Total suspended solid in shrimp-pond-water is mainly consists of shrimp feed excess which mostly contains protein, starch and lipid. Bacteria might degrade the feed particles into smaller ones using their extracellular enzymes. Proteases hydrolyze the smaller peptides and amino acids, facilitating partial absorption.

Protease and amylase activities of DA 5.2.3 isolate were higher than that of L5 isolate even though the PI values of the isolates were similar. This result implied that the PI value was not always correlated with enzyme activity in broth culture. In addition, the L5 isolate could decrease total suspended solid values by more than that of DA 5.2.3 isolate. This result might be arise because enzyme activities were measured using different substrates.

Alpha-amylase of the DA 5.2.3 isolate reached the maximum activities at the end of logarithmic growth phase, which was at 24 h from start of incubation. The activity was decreased sharply as the incubation time prolonged up to 48, 72 or 96 h. Cordeiro *et al.* (2002) reported that a maximum α -amylase production of a thermophilic *Bacillus* sp. strain SMIA-2 was reached at 48 h or at the late stage of logarithmic growth phase in basal medium supplemented with 0.5% (w/v) soluble starch. The activity was remained relatively constant up to 96 h. Another study by Santos and Martin (2001) found that α -amylase of moderately thermophilic *Bacillus* sp. was increased the production between 18-48 h of incubation in medium containing 1% (w/v) maltose as the carbon sources. The variations of exoenzyme synthesis among these three isolates are could be expected because bacteria were grown in different substrates.

Protease activity produced by the L5 and the DA 5.2.3 isolates reached maximum activity at 48 h or at the beginning of the stationary growth phase. Then, it decreased gradually as the time of incubation was extended. The length incubation times of maximum protease production of by *Bacillus* strain was reported by some researchers, as being from 24 to 120 h from start of incubation (Chu *et al.* 1992; Mabrouk *et al.* 1999; Beg and Gupta 2003). Genckal and Tari (2006) stated that decreasing enzyme activity with prolonged time, could occur because of hydrolysis of the enzyme by proteases.

Based on all the data of this study, the DA 5.2.3 isolate has potential application to maintain water qualities in shrimp ponds. Based on molecular identification, this isolate was similar with *Bacillus cereus* XHJ-2-6. So far, there is no information about using this strain as probiotic in shrimp aquaculture. Therefore, it will be a great challenge to explore its characteristics further for probiotic development in aquaculture industry.

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