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Potency of Bacillus cereus WPL 415 to Increase Crude Protein and Decrease Crude Fiber of Animal Feed Stuff

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Abstract

This research aims to identify isolate as a probiotic candidate derived from liquor rumen of local beef cattle and to know the ability of isolates as biofermentor on basal feed to the changes in the nutrient value. The selected samples were obtained from a slaughterhouse in Surabaya. This study consisted of two stages. The first stage was the identification of bacteria through the test of morphology, Gram staining, biochemical, resistance to acidity and 16S rDNA sequencing. The second stage was a test of the ability of the isolates on the nutrient of basal feeds by fermentation for three days in an aerobic condition. Based on the findings of the first phase, it has been identified that probiotic bacterium rods, motility positive, Gram-negative, have viability at pH 2 and pH 3 for 90 minutes and 24 hours and have the ability to ferment lactose, sucrose, galactose, ribose, cellobiose and xylose. Furthermore, based on test results of 16S rDNA sequencing, the probiotic bacterium was identified as *Bacillus cereus* WPL 415. Based on the research results at the second stage, *Bacillus cereus* WPL 415 at doses of 0.25% and 0.5% could improve the nutrient content of the basal feed. The results of the proximate analysis revealed that there was an increase in crude protein content of 6.78% until 8.12% compared to the control and was able to lower the crude fiber content of 15.19% and 17.40% compared to the control. Based on these results it can be concluded that *Bacillus cereus* WPL 415 from local beef cattle can be used as a probiotic candidates to improve the quality of animal feed.

Keywords: *Bacillus cereus,* probiotic, crude protein, crude fiber.

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1. Note

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2. Introduction

Probiotics are beneficial living microorganisms, either mono culture or mixed cultures that if applied to humans and animals will provide beneficial effects for the host by improving the properties of the indigenous flora, improving the health status of man or animals [1] and have the ability to modulate the balances and activities of the gastrointestinal (GI) microbiota [2]. Probiotic should be able to stimulate growth, improve feed conversion ratio and inhibit enteropathogens, without causing any undesirable effect. In the application process, probiotics must survive the stress produced during manufacturing, storage and administration at farm conditions [3]. Several strains have been used as probiotics i.e. *Lactobacillus, Pediococcus, Bacteroides, Bifidobacterium, Bacillus, Streptococcus* and *Escherichia coli,* alone or consortiated [4]. Based on the results of characterization, it was identified that *Bacillus* species *(Bacillus cereus, Bacillus clausii, Bacillus pumilus)* had potential as a probiotic based on the ability of colonization, immunostimulant, and antimicrobial activity [5]. *Lactobacillus plantarum* and *Bacillus spp.* spores have been reported to decrease the amount of Vibrionaceae in rotifers fed with these additives, and subsequently increase weight and survival of turbot larvae [6]. Although some strains of Bacillus species have been used as probiotics, but the information related to the advantages of using Bacillus have not been widely reported, thus, this study aims to carry out the exploring bacteria as a probiotic candidate sourced from isolated indigenous of local beef cattle from slaughterhouses in Surabaya.

3. Materials and Methods

The materials used are fresh gut of local cattle from abattoirs, alcohol 70%, medium selective MRSB (de Man Rogosa Sharpe Broth), MRSA (de Man Rogosa Sharpe Agar) (OXOID), medium Luria Bertani (LB) (MERCK), Gram stain (Crystal Violet, Lugol, alcohol-acetone, and safranin), physiological NaCl, 0.1 N HCl. **Sample Preparation**. The part of

intestinal organs were rinsed with sterile distilled water and crushed using a mortar and weighed as much as 1 g and put into a sterile physiological NaCl solution and diluted with multilevel dilution (10^{-1} - 10^{-6}). A total of 1 ml of 10^{-6} dilution was inoculated on MRSA (Man Ragosa Sharpe Agar) medium, then incubated for 24-48 hours at 37°C. **Purification of Bacteria Probiotic Candidate**. Purification was done by selecting a colony on the surface and then inoculated on the surface of MRSA (Man Ragosa Sharpe Agar) medium with scratch method to obtain a separate colony. It was incubated at 37°C for 2x24 hours. The purification step could be done 2-3 times to obtain pure isolates. Furthermore, the isolate was inoculated on MRSA medium slant as stocks for further testing. The observation of cell morphology was done by using the Gram stain.**Test of Resistance on Acidity (pH).** The resistance test on acidity was done using MRS broth medium supplemented with 0.1 N HCl to obtain pH 2-3 (according to the pH of the stomach). As much as 1 ose each bacterial isolates was taken from the stock culture and was inoculated on MRSB-HCl medium. After that, it was incubated for 2x24 hours at 37°C. If there was a growth of bacteria on MRSB-HCL medium, it showed positive results, and it showed negative results if there was no growth of bacteria on the MRSB-HCL medium. **Identification of the gene encoding 16S rDNA.** DNA isolation was using the method of Ausubel [7]. **The second stage.** In the second phase of the study was carried out a test of *B.cereus* WPL 415 ability against basal feed through the fermentation process. The study was divided into three treatments with each of eight repetitions. The treatment consisted of: Po: Po: feed without *B.cereus* WPL 415, P1: feed + 0.25% *B.cereus* WPL 415, and P2: feed + 0.50% *B.cereus* WPL 415. The fermentation of basal feed was done by adding a solution of inoculant appropriate treatment dose, then dissolved in a 3% drop and 20% non-chlorine water. After the solution was mixed homogeneously, sprayed on basal feed. Fermentation was done under anaerobic conditions using a plastic bag as a silo for 3 days. After the fermentation period ended, the plastic was opened, then proximate analysis was performed to determine changes in the nutritional content of crude protein and crude fibre. Data were analysed by analysis of variance. If the results were significantly different ($P < 0.05$) between treatments, the analysis was proceeded by Duncan's multiple range test [8].

4. Results and Discussion

Based on identification results, the isolate obtained from the small intestine of cattle, had the following characteristics: a rod-shaped cells, Gram negative and positive

Table 1: Biochemistry Test Results of WPL 415.

Biochemistry Test	reaction	Biochemistry Test	reaction
Lactose	+	Galactose	+
Sucrose	+	Cellubiose	+
Gluconaet	+	Rafinose	+
Ribose	+	Manitol	+
Xylose	+	Ramnose	-
Arginine	-	Esculine	+
Arabinose	-		

motility. The results of biochemical tests isolates obtained in this study demonstrated the ability of fermentation as listed in Table 1.

The arrangement of obtained nucleotide isolates, further was identified with the program BLAST (Basic Local Alignment Search Tool) in www.ncbi.com and isolates obtained which had some similarities with the arrangement of nucleotide similarity level 92% -88%. (Bacillus cereus ATCC 14579, identity 92%, sequence ID ref|NC_004722.1; Bacillus cereus Rock4-18, identity 92%, sequence ID ref|NZ_CM000735.1; Bacillus cereus AH621, identity 92%, sequence ID ref|NZ_CM000719.1; Bacillus megaterium DSM319, identity 89%, sequence ID ref|NC_014103.1; Bacillus licheniformis ATCC 14580, identity 88%, sequence ID ref|NC_006270.3; Bacillus hemicellulosilyticus JCM 9152, identity 88%, sequence ID ref|NZ_BAUU010000088.1; Bacillus amyloliquefaciens DSM7, identity 88%, sequence ID ref|NC_014551.1; Bacillus subtilis subsp. subtilis str. 168, identity 88%, sequence ID ref|NC_000964.3; Bacillus subtilis subsp. spizizenii TU-B-10, identity 88%, sequence ID ref|NC_016047.1; Bacillus cellulosilyticus DSM 2522, identity 88%, sequence ID ref|NC_014829.1)

This research showed new isolate namely *Bacillus cereus* WPL 415. Based on the research results of Navinchandran, had been identified through sequencing of 16S rRNA, a probiotic bacterium *Bacillus cereus* from the gut of wild shrimp *Penaeus monodon* [9]. The probiotic bacterium had antagonistic activity against pathogenic bacteria in shrimp as well as having the ability to produce extracellular enzymes. Probiotic *B. cereus* at a concentration of 0.4% / 100 g of feed was efficient in stimulating the growth (specific growth rate / SGR of 4.40 ± 0.179% and a better feed conversion ratio / FCR of 1.27 ± 0081) and immunity (total count haemocyte, lysozyme activity, plasma protein concentration and bactericidal activity) in shrimp.

Table 2: Viability of the bacteria *Bacillus cereus* WPL 415 at pH 3 and pH 4.

Viability of the bacteria *Bacillus cereus* WPL 415			
Time	MRS Agar (control) CFU/ml	MRS Agar pH 3 CFU/ml	MRS Agar pH 4 CFU/ml
90 minutes	1.4×10^8	1.0×10^8	2.1×10^8
	2.1×10^8	1.2×10^8	2.0×10^8
24 hours	7.5×10^8	1.0×10^8	3.6×10^8
	6.6×10^8	0.4×10^8	4.8×10^8

Among the various species of probiotics, those belong to the genus of *Bacillus* which had the advantage that, due to their capacity to sporulate, they survive at ambient temperatures as well as during desiccation by methods that involve; moderate heating, such as spray dryers, avoiding the use of lyophilization or other expensive technologies [10]. *B. cereus* strains were shown to persist in the mouse gastrointestinal tract for up to 18 days post administration, demonstrating that these organisms had some abilities to colonize. The spores of one *B. cereus* strains were extremely sensitive to simulated gastric conditions and simulated intestinal fluids [11].

Bacillus is able to grow on various kinds of sugar, these isolates also has a cellulolytic activity shown on its ability to degrade trinitrophenyl-carboxymethyl cellulose and growth on medium containing glucose cellobiose or produce the largest cellulolytic activity. Cellulolytic activity is not generated until the stationary phase of growth. Maximum cellulolytic activity test occurs at pH 4.8 and a temperature of 58 °C [12].

The research results in Table 2 indicated that the isolates *of Bacillus cereus* was able to sustain life in the acidic conditions of pH 3-pH 4. It was shown from the comparison with the controls, which in 90 minutes showed the viability of control about 1.4×10^8 - 2.1×10^8 CFU / ml. On the condition of acid pH 3 for 90 minutes, *Bacillus cereus* isolates demonstrated the viability of 1.0×10^8 - 1.2×10^8 CFU / ml, whereas at pH 4 for 90 minutes showed the viability of $2.0 \times 10^{8-}$ 2.1×10^8 CFU / ml. Later in the control condition for 24 hours, *Bacillus cereus* isolates demonstrated the viability of 7.5×10^8 - 6.6×10^8 CFU / ml, whereas at pH 3 for 24 hours demonstrated the viability of $0.4 \times 10^{8-}$ 1.0×10^8 CFU / ml. On the condition of acid pH 4 demonstrated the viability of $3.6 \times 10^{8-}$ $4.8. \times 10^8$ CFU / ml.

Resistance to acidity test results showed that isolates of *Bacillus cereus* was considered able to survive through the digestive tract system that had a low pH conditions, so as to reach the intestine to be able to do activities to maintain the balance of microflora. *Bacillus cereus* has the ability to survive in the intestinal tract and during manufacturing, such as interaction with enteropathogens, resistance to heat and to variation of pH.

Table 3: *B. cereus* fermentation influence on the levels of crude protein and crude fibre of feed.

Treatment	Crude Protein ± SD	Crude Fiber ± SD
Po (0% *B.cereus* WPL 415)	17.99^{a} ±0.07	6.78^{a} ±0.68
P1 (0.25% *B.cereus* WPL 415)	19.21^{b} ±0.02	5.60^{b} ±0.21
P2 (0.50% *B.cereus* WPL 415)	19.45^{b} ±0.5	5.75^{b} ±0.25

[a] different superscripts in the same column, shows that there were significant differences (p <0.05).

Variations in pH of the gastrointestinal tract could affect the viability of the isolate as a probiotic candidate. Bacillus genus have strong adaptability to diverse conditions and that several species produce highly resistant spores; they have been isolated from fish [13;14]. Based on the results of statistical analysis, the use of *B. cereus* WPL 415 in the fermentation basal feed showed significant differences among treatments on the content of crude protein and crude fibre (Table 3).

The results of the proximate analysis the crude protein content was the lowest for the Po treatment (control, without the use of *B. cereus* WPL 415), while the use of 0:25% *B. cereus* WPL 415 and 0.5% crude protein were able to increase the content of 6.78% until 8:12% compared with control. The highest results of the proximate analysis of crude fibre content was at Po treatment (control, without the use of *B. cereus* WPL 415), while the use 0.25% *B. cereus* WPL 415 and 0.5% were able to lower crude fibre content of 15.19% to 17:40% compared to control patients. The results of this study indicate that WPL 415 *B. cereus* was able to increase the nutrient of the forage. The increase in crude protein content was due to the increased biomass of single cell protein *B. cereus* WPL 415. In the fermentation process, nutrients were available for the media used for the breeding biomass of *B. cereus* WPL 415, thus, it increased the number of microbe biomass, in which the increase was detected from the results of proximate analysis of crude protein.

The use of *B. cereus* WPL 415 in the fermentation basal feed could also reduce the content of crude fibre. It was supported by the test results that demonstrates the ability of isolates to ferment crude fibre, namely xylose and cellobiose. Cellulose fraction was the biggest component of a constituent of plant cell walls that were very difficult and even could not be digested by monogastric digestive enzymes, so that the cellulose must be broken down first into low molecular weight compounds such as mono, di and tri saccharides. The degradation involved a complex of cellulase enzymes produced by microbes are endo-beta-glucanase and beta-glucosidase.

Cellulase enzyme is a complex enzyme consists of a group of enzymes that work synergistically to degrade cellulose i.e. 1, 4-β-D-glucan-4-glucanohydrolases or endoglucanase; 1, 4-β-D-glucan glucanohydrolases or exoglucanases and β-glucoside glucohydrolases or cellobiase. Endoglucanase enzyme randomly cut the internal amorphous on the chain of 1, 4-β polisaccharides cellulose into cellulo-oligosaccharides. Exoglucanases enzyme take a role in glucose unit at the polar end of the reduction or non-reduction of the chain of cellulo-oligosaccharides produces Cellobiose (disaccharide). The β-Glucosidases enzyme hydrolyse Cellobiose into glucose [15]. Supplementation of 0.20% -0.40% rumen bacterial culture cellulolytic isolates buffalo can improve the body weight gain and feed efficiency of ducks. This is due to the addition of a bacteria culture acts as probiotics can stimulate the synthetic enzyme digestion enhancing the utilization of nutrients [16].

Based on the results of the study [17], the use of cow's rumen fluid was very potential as inoculant that contained of high nutrients and ready fermentable microbial and fibre degrading enzymes. The use of rumen fluid was capable of producing an inoculant with high nutrient and microbiology that were effective to be used as a starter. The high population of microbial inoculant and the support by the substrate degradation ability was high, and the high activity of cellulose and xylanase enzymes had been able to decrease crude fibre content of the ration so that the nutrient of the ration fermented inoculants' quality produced increases. The improvement quality of the nutrient of the fermented feed gave a positive response to the increase in the digestibility [18]. The results of other studies indicate the existence of extracellular products produced by Bacillus sp which indicates strongly inhibited the growth of *Aeromonas hydrophila* and *Vibrio alginolyticus* isolated from diseased fish. APIZYM Enzyme assays showed that both bacteria have esterase lipase, leucine arylamidase, acid phosphatase, lipase and Naphthol-AS-BI-phosphohydrolase activities [19].

5. Conclusion

Based on these results it can be concluded that *Bacillus cereus* WPL 415 from local beef intestine can be used as a probiotic candidates to improve the nutrient value of animal feed stuff.

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