Advances in Biological Sciences Research, volume 22
7th International Conference on Biological Science (ICBS 2021)

Lipolytic and Proteolytic Activities of Fibrolytic Bacteria from Buffalo (*Bubalus bubalis*) Rumen

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ABSTRACT
The use of plant-based feed ingredients in aquaculture is expected to reduce feed costs. However, plant ingredients generally have a low protein content and are difficult to be digested by monogastric animals. The fermentation process is proven to increase the digestibility and nutritional value of plant ingredients. Previous research succeeded in isolating fibrolytic bacteria from buffalo rumen. The ability of microbes to degrade various macromolecules is crucial for their application in feed fermentation. Therefore, this research was conducted to determine the lipolytic and proteolytic activities of fibrolytic bacteria from buffalo rumen. A total of 30 fibrolytic bacterial isolates were tested for their lipolytic and proteolytic activities. The proteolytic activity was determined based on the ability to produce clear zones on the skim milk agar (SMA) medium. Lipolytic activity was determined by using a rhodamine B-olive oil agar medium. The results showed that all fibrolytic bacterial isolates had proteolytic and lipolytic activities. The molecular identification results based on the 16S rRNA gene sequence showed that the bacterial isolates were members of the phylum Firmicutes, namely *Bacillus*, *Paenibacillus* and *Exiguobacterium*; phylum Proteobacteria, namely *Acinetobacter*, and *Klebsiella*; and the phylum Bacteroidetes, namely *Chryseobacterium.*

Keywords: *Buffalo rumen, Fibrolytic bacteria, Lipolytic, Proteolytic.*

1. INTRODUCTION

Feed cost is the largest component of production cost in aquaculture, reaching 50-60% of total production costs [1]. Reduction of feed costs will reduce production costs and increase profits significantly. The main component of fish feed that is needed in large quantities and is most expensive is the source of protein. Fish require 2-3 times more protein than other vertebrates or range from 30-55% dry weight of feed [2]. Most commercial feeds on the market today use the fish meal as a protein source. The use of plant-based protein as a substitute for fish meals is expected to reduce feed costs. However, due to the relatively low protein content, low palatability, the presence of anti-nutritional factors (ANFs), deficiency of various essential amino acids, and low nutrient digestibility, the use of plant-based protein sources is very limited in aquaculture [3][4]. Feed fermentation has been carried out to improve digestibility [5], nutritional value, and feed efficiency of plant-based feeds [4].

During the fermentation process, carbohydrate and protein polymers are broken down into water-soluble low molecular weight peptides, oligosaccharides, and monosaccharides [5]. In addition, fermentation can trigger the production of antioxidants and antibacterial compounds that improve fish health [6].

Previous studies have successfully isolated 34 cellulolytic bacteria from the buffalo rumen, a ruminant that has superior ability in adapting to the diversity of plant-based feed [7]. Those bacterial isolates were known to have the ability to grow on palm kernel meal media. The main carbon source in palm meal comes from plant cell walls, which contain a lot of structural polysaccharides, including hemicellulose (61.5%) and cellulose (11.6%) [8][9]. In addition to polysaccharides, palm meal also contains a large amount of protein and lipid. Crude protein content in palm meal ranges from 14-21% while total lipids range from 8-17% [10]. Based on this, in addition to the cellulose and mannan-degrading capabilities, those fibrolytic bacteria grown in palm kernel media may be able to utilize protein or lipid as a carbon source or have proteolytic and lipolytic activities. Therefore, the purpose of this study was to evaluate the lipolytic and proteolytic abilities of fibrolytic bacterial which have been isolated from buffalo rumen in a previous study (7).

2. MATERIALS AND METHODS

2.1. Microorganism

Fibrolytic bacterial isolates were obtained from previous research [7]. Those bacterial isolates were known to grow on palm kernel meal media.

2.2. Media

Culture regeneration and inoculum preparation used Nutrient Broth (NB) and Nutrient Agar (NA) media. Screening for proteolytic activity used protease skim milk agar (SMA) media containing: 0.1% peptone, 0.5% NaCl, 2% agar, and 2% Skim Milk (Oxoid). Screening for lipolytic activity used Rhodamine B-olive oil-agar (ROA) media with the composition: 0.8% NA, 0.4% NaCl, 2% agar, 31.25 mL/L Olive oil, and 500 µL 0.01% rhodamine B solution in distilled water.

2.3. Screening for proteolytic and lipolytic activity

Inoculum preparation was carried out by inoculating bacterial isolates into NB media and incubated at 34 ^{o}C with 120 rpm agitation for 18 hours. A total of 10 µL of inoculum was taken with a micropipette, dripped on the surface of SMA media and then incubated at 34 ^{o}C for 18 hours. Proteolytic activity was indicated by the formation of a clear zone around the colony [11].

The culture from NA agar slant (18 hours of age) was taken with the nidle and then inoculated on ROA media streak plate technique. Incubation was carried out at 34 ^{o}C for 24 hours. Lipolytic activity was indicated by the presence of a red-orange color under UV light with a wavelength of 350 nM [12].2.4. Identification of bacterial isolates based on 16S rRNA gene sequence

Genomic DNA extraction was done by using Presto ™ Mini gDNA Bacteria Kit (Genaid, Taiwan) according to the manufacturer's guide. Amplification of 16S rRNA gene was conducted with universal primers for bacteria, namely 63F (5' - CAGGCCTAACACATGCAAGTC-3 ') and 1387R (5' - CCCGGGAACGTATTCACCGC-3') [13]. The PCR reaction with a total volume of 50 ml, consisting of 21 µL ddH2O, 25 µL of 2x MyTaqTM HS Red Mix, 1 µL of 10 pmol 63F primers, 1 µL of 10 pmol 1387R primers, and 2 µL DNA template. PCR was done using a Thermo Cycler (Applied Biosystem) machine with the following steps: 3 minutes of pre-PCR at 95˚C, 30 cycles (denaturation at 94˚C for 15 seconds, annealing at 56˚C for 15 seconds, and elongation at 72˚C 30 seconds), and final elongation at 72˚C for 2 minutes. PCR product purification and sequencing were conducted by 1st BASE (Singapore). 16S rRNA gene sequence was compared with data available at GenBank by using the BLAST program of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

3. RESULT AND DISCUSSION

3.1. Screening for proteolytic and lipolytic activity

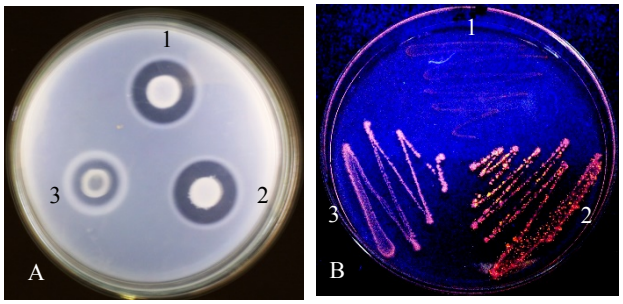

Figure 1. Isolate BR23 (A1), BR24(A2), and BR25 (A3) on skim milk agar media, age 18 hours. The clear zones around the colony indicate proteolytic activities. Isolate BR25 (B1), BR34 (B2), and BR30 (B3) in rhodamine B-olive oil media, aged 24 hours, under UV exposure at 350 nM wavelength. The glowing orange color indicates lipolytic activity.

A total of 30 fibrolytic bacterial isolates were tested for their lipolytic and proteolytic activities. Proteolytic activity was indicated by the formation of a clear zone around the colony (Figure 1A). Those bacterial isolates

Table 1. Screening results for Proteolytic and lipolytic activities of fibrolytic bacterial isolates from buffalo rumen

Code of Isolate	Proteo-lytic activity (dz/dz)	Lipo-lytic Activity	Code of Isolate	Proteo-lytic activity (dz/dz	Lipo-lytic Activity
BR1	0.48	√	**BR16**	0.50	√
BR2	0.48	√	**BR17**	0.45	√
BR3	0.50	√	**BR18**	0.50	√
BR4	0.48	√	**BR19**	0.47	√
BR5	0.50	√	**BR20**	0.45	√
BR6	0.55	√	**BR23**	0.50	√
BR7	0.55	√	**BR24**	0.50	√
BR8	0.63	√	**BR25**	0.67	√
BR9	0.50	√	**BR26**	0.58	√
BR10	0.52	√	**BR27**	0.52	√
BR11	0.48	√	**BR28**	0.90	√
BR12	0.55	√	**BR29**	0.89	√
BR13	0.48	√	**BR30**	0.53	√
BR14	0.43	√	**BR33**	0.45	√
BR15	0.55	√	**BR34**	0.43	√

produced extracellular proteases that hydrolyze proteins in the media to form a clear zone around the colony. Lipolytic activity was indicated by the presence of an orange fluorescent under UV light at 350 nM wavelength (Figure 1B). Lipid hydrolysis produces free fatty acids which will interact with Rhodamine B to produce an orange, fluorescent complex with an excitation wavelength of 350nm [14] [15]. The orange color intensity increased along with the increase of lipolytic activity [16].

The results showed that all fibrolytic bacterial isolates had lipolytic and proteolytic abilities (Table 1). Ruminants are a group of herbivorous animals that use rumen fermentation to help the process of digestion of feed ingredients. Therefore, ruminants build symbiosis with microbes, including bacteria, protozoa, fungi, archaea, and bacteriophages, in their rumen [17]. Rumen microbes produce a wide variety of enzymes, including cellulase and hemicellulase [18], lipase [19], and protease [20] [21], that play an important role in feed digestion process. Among the various types of ruminal

Table 2. Identification results based on 16S rRNA gene sequences [7].

Code of isolate	Description	Per. Ident	Query cover	E Value	Accession
BR1	*Bacillus aryabhattai*	99.19	99	0.0	MN181350.1
BR2	*Bacillus cereus*	99.49	99	0.0	MN752435.1
BR3	*Bacillus cereus*	99.75	100	0.0	MK045762.1
BR4	*Bacillus aryabhattai*	99.71	99	0.0	KY622236.1
BR5	*Bacillus cereus*	98.81	99	0.0	LC189362.1
BR6	*Bacillus cereus*	99.91	100	0.0	MK045762.1
BR7	*Bacillus cereus*	100	100	0.0	KU510086.1
BR8	*Acinetobacter baumannii*	99.19	100	0.0	MN749520.1
BR9	*Bacillus cereus*	99.13	98	0.0	MH399242.1
BR10	*Bacillus thuringiensis*	99.37	99	0.0	MK026865.1
BR11	*Bacillus megaterium*	99.89	99	0.0	MK934384.1
BR12	*Bacillus cereus*	99.32	99	0.0	KY777580.1
BR13	*Bacillus cereus*	99.73	99	0.0	KP813794.1
BR14	*Exiguobacterium acetylicum**	98.98	99	0.0	MN650223.1
BR15	*Acinetobacter baumannii*	99.83	99	0.0	CP042931.1
BR16	*Bacillus paramycoides**	99.58	99	0.0	MH734764.1
BR17	*Bacillus megaterium*	100	100	0.0	MK934384.1
BR18	*Bacillus zanthoxyli*	99.41	100	0.0	NR_164882.1
BR19	*Bacillus cereus*	100	99	0.0	MN733060.1
BR20	*Bacillus Sp.*	96.58	100	0.0	MK490763.1
BR23	*Klebsiella quasipneumoniae**	100	100	0.0	CP045641.1
BR24	*Chryseobacterium bernardetii*	96.69	100	0.0	CP033931.1
BR25	*Paenibacillus polymyxa**	98.57	99	0.0	KR780413.1
BR26	*Acinetobacter baumannii*	98.86	100	0.0	CP040041.1
BR27	*Bacillus cereus*	99.66	100	0.0	KY777580.1
BR28	*Bacillus koreensis*	99.75	100	0.0	MH169307.1
BR29	*Bacillus koreensis*	97.95	99	0.0	MK618615.1
BR30	*Acinetobacter baumannii**	99.57	100	0.0	CP042556.1
BR33	*Klebsiella quasipneumoniae*	99.01	99	0.0	CP045641.1
BR34	*Klebsiella pneumoniae*	99.57	100	0.0	CP042620.1

microorganisms, the bacteria are considered to be most active in lipolysis [22].

The ability of bacteria to break down a wide variety of macromolecules is very beneficial for its application to the feed fermentation process. The main purpose of fermentation is to break down complex macromolecules into simpler molecules and increase the digestibility and nutrient availability of feed ingredients. Feed fermentation could increase nutrient efficiency and nutritional value of aquafeed, has beneficial effects on gastrointestinal tract (GIT) ecosystems and morphology, minimize colonization of GIT pathogens and improve immune response [4]. In addition to the ability to hydrolyze polysaccharides, proteolytic and lipolytic abilities are important in improving aquafeed digestibility. The main applications of proteases in animal nutrition are during the processing of feed ingredients or direct application of exogenous proteases as supplements to feed [23]. Protease breaks down proteins into simple peptides that are more available and easier to digest. Fermentation of plant-based feed could increase protein solubility up to 2-fold [24] Dietary lipids play a crucial role in the fish diet, both as energy and essential fatty acid resource of energy and essential fatty acids that are essential for fish growth but cannot be synthesized on their own [25][26]. Supplementation of lipase significantly improved the gut and hepatopancreas lipase activity, feed conversion ratio, and growth performance of fish fed with palm oil diet [27].

Bacterial isolates that have not been identified in previous studies were further identified based on the 16S rRNA gene sequences. The identification results showed that almost all isolates had similarities to the database in the gene bank with similarities above 98% except BR 24 which was closely related to *Chryseobacterium bernardetii* with a similarity rate of 96.58% (Table 2). A total of 7 isolates belonged to the phylum proteobacteria, an isolate belonged to the phylum bacteriodates, and 22 isolates belonged to the phylum firmicutes.

In general, Firmicutes, Bacteroidetes, and Proteobacteria are the dominant bacteria in cow rumen of various ages [28]. Bacteroidetes and Firmicutes were the dominant phyla in buffalo and cow rumen but in buffalo rumen, the abundance of firmicutes was higher than in cow rumen. The population of bacteriodates and firmicutes in the rumen involve in the degradation of organic matter into simpler forms [29]. The higher abundance of firmicutes in buffalo rumen indicates the higher ability to digest plant-based feed [30]. The higher abundance of firmicutes in the rumen is also associated with carbohydrate fermentation in the rumen [31], feed efficiency, and daily weight gain [32].

These fibrolytic bacteria had the potential to be applied to the fermentation of plant-based feed ingredients because apart from being able to hydrolyze fibre, they were also able to hydrolyze proteins and lipids. The ability to break down various kinds of macromolecules is very beneficial in its application in the feed fermentation process. However, further research is needed regarding its effectiveness and safety for the animal.

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