Extracellular Enzyme Production of Probiotic *Bacillus* JAQ04 and *Micrococcus* JAQ07 isolated from tiger grouper (*Epinephelus fuscoguttatus*)

M. Z. Nora Azirah, I. Marini, K. Murni, S. A. Harmin, M. Y. Ina-Salwany

***Abstract*—**Tiger grouper has been identified as a good market price and most desired in the live fish trade market species especially in Southeast Asia region. However, intensive grouper aquaculture often triggering diseases by bacteria pathogen that leads to infectious diseases. Thus, to hinder this infectious disease, promising probiotic bacteria successfully isolated from intestine Tiger grouper juvenile. Previous studies showed that this bacterium has been shown to produce antibacterial activity against pathogenic marine bacteria *Vibrio alginolyticus*. Thus, this study aims to further identify the characteristic of Probiotic *Bacillus* JAQ04 and *Micrococcus* JAQ07 *in-vitro*. In this study the bacteria were screened for their extracellular enzyme production of protease, amylase and lipase. These bacteria may play a role in inhibiting the pathogen by production of extracellular enzyme and improve the feed digestion. The enzymes were shown positive for protease, amylase and lipase which exhibit the clear zone on skim milk agar, starch agar and spirit blue agar. However, *in vivo* study needs to be done to further confirm the enzymatic activity of the isolates in inhibiting of pathogen and improve feed digestion.

***Keywords*—**Amylase, *Bacillus*, *Micrococcus*, Lipase, Protease

I. Introduction

The information regarding the enzyme producing bacteria is still scared. However, previous studies identified that the bacterial flora of the gastrointestinal tract with diverse enzymatic potential play a vital role in major part of and their role in the pathogenesis of infectious diseases as well as to improve the metabolism of the host animal [1]. Apart from the beneficial effect of bacteria to *Artemia* growth through the contribution of extra nutrients, they are also believed rich in exogenous enzymes that help in digestion and absorption process in gut larvae or food organisms by breaking down of food to smaller particle [2] - [3]. Furthermore, marine organism which is saline in nature chemically closer to the human blood plasma could provide microbial product, particularly enzyme that safe and having no or less toxic or side effect when used for therapeutics application even to human [4]. Further, it is believed that the marine organism has a diverse range of enzymatic activity that capable catalyzing various biochemical reactions. Thus, this study was aimed to identify the extracellular enzyme production by probionts *Bacillus* JAQ04 and *Micrococcus* JAQ07 isolated from tiger grouper (*Epinephelus fuscoguttatus*).

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II. Materials And Methods

A. Isolation of Bacteria Strains

The pure culture *Bacillus* JAQ04 and *Micrococcus* JAQ07 were collected from Department of Agriculture, Universiti Putra Malaysia. The samples placed in glycerol stock were plated on marine agar plates. The plates were incubated overnight at 30°C. After incubation the colony checked for purity according to *Bergey's manual Systemic Bacteriology*. Fresh bacteria cultures were used to screen for enzyme production.

B. Protease activity

For determination of protease activity, the media were prepared according to previous studies by dissolving 10g skim milk in 90 ml distilled water and 3g of agar was dissolved in 97 ml distilled water. After autoclaving both solutions separately, they were then mixed together and dispensed in a petri dish. The samples were inoculated on skim milk agar plates and incubated 24 hours at 30°C. A zone of clearance around colony indicated production of protease [5].

C. Amylase activity

The intensity of extracellular enzyme production of pure culture was obtained by streaked 24 hours fresh culture approximately 5mm diameter at the center of selective media starch agar. Following incubation, the plates were flooded with 1% Lugol iodine solution. Formation of transparent around the colony indicated amylase activity [6].

D. Lipase activity

For assaying the lipase activity, the spirit blue media were supplemented with olive oil emulsion according to manufacturer (HiMedia). The plates then were inoculated with the test organism. The plates were inverted and incubated for 24 hours. Lipase activity was noted by the appearance of an iridescent sheen (oil and water) that can be seen when the plate held at an angle to a light source.

III. RESULTS AND DICUSSION

The zone inhibition (total diameter minus the diameter of the colony) was considered proportional to the enzymatic activity. Qualitative extracellular enzyme activity was observed by the appearance of halo diameter in mm around the colony was presented as scores as follows: -, nil (no halo); +, low (1-4 mm halo); ++, (5-8 mm halo); +++, high (9-12 mm halo); +++, very high (≥13 mm halo). The intensity of extracellular enzyme production by the bacterial strains isolated from the gut of *Epinephelus fuscoguttatus* was assayed qualitatively (Table 1) in which each '+' indicates a zone diameter of 4 mm.

Among the isolates, the probionts JAQ04 exhibit protease and amylase activity where moderate inhibition zone (6 mm) was observed around the colony while JAQ07 exhibited protease activity with the inhibition zone (7 mm). For lipase assay, both probionts were showed high intensity (10 mm) of extracellular enzyme production.

TABLE I
INTENSITY OF EXTRACELLULAR ENZYME PRODUCTION

Bacteria Strains	*Protease*	*Amylase*	*Lipase*
Bacillus JAQ04	++	++	+++
Micrococcus JAQ07	++	-	+++
Control	-	-	-

'+' sign indicates the intensity of enzyme production (zone diameter of 4 mm). ++++, very high; +++, high; ++ moderate; +, low; —, nil.

Fig. 1 Skim milk media showed inhibition zone around probionts JAQ04 (left) and JAQ07 (right) indicated the protease activity.

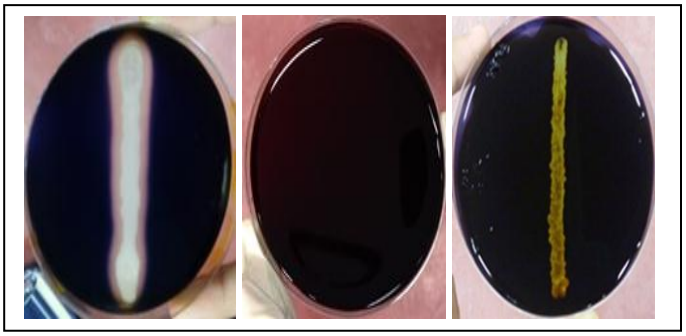

Fig. 2 Starch agar flooded with 1% iodine showed inhibition zone around probionts JAQ04 (left) while no inhibition zone showed in JAQ07 (right).

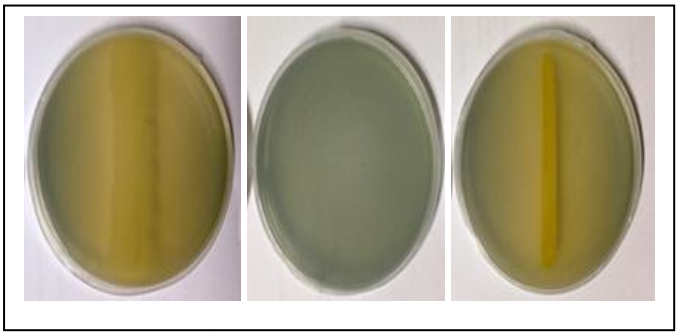

Fig. 3 Appearance of an iridescent sheen around probionts JAQ04 (left) and JAQ07 (right).

In the present study, an attempt was made to determine enzymatic activity on solid culture agar plates. *Bacillus cereus* JAQ04 is able to produce protease, amylase and lipase enzyme. This result is in agreement with the finding by previous study who reported that the bacterium *Bacillus cereus* able to produce extracellular enzyme protease, amylase, lipase, and phytase [7]. The study also revealed that the bacteria rendering antagonistic activity against shrimp pathogen, *Vibrio harveyi* and *Aeromonas hydrophilla*. Meanwhile, *M. luteus* revealed that unable to produce an amylase enzyme [8]. The finding was similar to the present study where negative amylase activity showed by *Micrococcus luteus* JAQ07. However, the further study of digestive enzymes is essential towards understanding the mechanism of digestion and how organisms adapt to changes in the nutritional environment.

IV. DISCUSSION

Apart from contribution of extra nutrients, enzymes are believed help in digestion and absorption process in gut larvae or food organism by breaking down food to smaller particle. Thus, the enzyme producing bacteria could improve in digestion which correlated with their feeding habits of the fish [9]. It has been suggested that microbiota have a positive effect on the digestive process of fish due to enzyme production by fish gut bacteria. Thus, many studies try to improve the fish production by application of enzyme producing bacteria. Shrimp administered with commercial

Bacillus were significantly increased survival due to the amylase, protease, and lipase digestive enzyme activity when compared to control (no probiotic administered) [10]. From the present study, we also found that the probionts *Bacillus* JAQ04 and *Micrococcus* JAQ07 isolated from the healthy gut of the tiger grouper capable producing extracellular enzymes protease and lipase. Proteolytic enzymes that hydrolyze casein allow the organisms to break down casein into smaller polypeptides, peptides, and amino acids that can cross the cell membrane and be utilized by the organism. Meanwhile, lipase enzyme capable of breaking down lipids. Many organic molecules are too large to enter the cell, so a lipase is released to break it down prior to cellular uptake that used by some bacteria for carbon and energy. However, *Micrococcus* unable to secrete exoenzymes a-amylase to degrade starch into subunits that can then be utilized by the organism. These probionts producing enzyme could give significant effect in fish aquaculture as a source of an enzyme which is more active and stable than plant and animal sources. In addition, the microorganism is an alternative source of enzymes because they can be cultured in large quantities in a short time by fermentation [11].

Furthermore, probiotic in aquaculture have shown to have several modes of action such as competitive exclusion for nutrition, production of inhibitory compound, improvement of water quality, enhancement of immune response of the host and enhancement of host nutrient through the production of supplemental digestive enzyme [12]. The previous study identified that the bacterial flora of the gastrointestinal tract with diverse enzymatic potential play a vital role in the pathogenesis of infectious diseases as well as to improve the metabolism of the host animal [1]. It is particularly because gram positive bacteria especially *Bacillus* does secrete a wide range of exoenzyme. Previous studies found that *Bacillus* secretes many enzymes that degrade slime and biofilms and allow *Bacillus* to enter and their antibiotic to penetrate the slime layer around gram negative bacteria [13]. The information generated from the present investigation might contribute to the utilization of this extracellular enzyme producing bacteria JAQ04 and JAQ07 in inhibiting *V. alginolyticus* in *Artemia* culture and against several fish pathogens (*V. paraheamolyticus*, *V. harveyi*, and *Aeromonas hydrophila*) [14] -[15].

Enzymes are produced in every living organism from higher animals and plant to unicellular form of life as they are essential for metabolic pathway. In animal, digestions of food are carried out by animal digestive system and by microorganism inhabit the intestinal tract. The pancreatic digestive enzyme has an essential role in the digestion, trypsin and chymotrypsin are the main pancreatic proteases, lipase is the major of pancreatic lipolytic enzyme, and amylase is known as the major pancreatic digestive enzyme for carbohydrate [15]. However, aquatic animal is lack of certain digestive enzyme during early development and even throughout their life. To encounter this problem, fish feed manufacturers are trying to improve the nutritional value of fish meal by supplementation of the enzyme. The addition of live microorganism to diets to produce an enzyme is possible in feed application. Thus, in large scale, commercial enzyme applications are rely on the enzyme produced by microbial fermentation technology. This make the enzyme producing bacteria *Bacillus* JAQ04 and *Micrococcus* JAQ07 are possible used for industrial purpose especially in fish feed production. However, *In vivo* studies need to be done to further evaluate the role of enzyme producing bacteria *Bacillus* JAQ04 and *Micrococcus* JAQ07 in pathogenesis and digestive system of fish.

V. Conclusion

The data might contribute a possible nutritional strategy for fish nutritionist to utilize enzyme producing bacteria as a probiotic and as a cost effective in aqua feeds. However, the influence of these enzyme producing bacteria on the fish productivity is not known and requires further investigation

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