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Extracellular Amylase Activity of Amylolytic Bacteria Isolated from Quail's (*Coturnix japonica*) Intestinal Tract in Corn Flour Medium

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Abstract: Amylolitic microorganisms have capability of producing amylase which is an important enzyme in industries such as paper, textile and food industries. These microorganisms are found as a normal intestinal microflora in poultry's digestive tracts including quail (*Coturnix japonica*), especially with carbohydrates as a most nutrient in their ration. Corn becomes most important feed for quail because it has high energy related to its high amylum content. Amilolytic microorganisms in the digestive tract digest amylum by producing an extracellular enzyme (amylase) which breakdown amylum into simpler molecules facilitating for its absorbtion in the digestive tract. This research aimed to isolate amylolytic bacteria from quail's digestive tract, to determine their growth and amylolytic activity in corn flour medium. Isolation was done by using Starch Agar medium, then amylolytic activity was indicated based on intensity of clear zone formation in the media and by amylase assay using DNS method in Starch Broth medium. Growth curve and amylase assay was carried out in 2% Corn Flour Broth medium. Data were subjected to analysis of Pearson correlation. The results showed that six isolates called as BAP1, BAP2, BAP3, BAP4, BAP5 and BAP6 were found. BAP6 isolate showed the widest clear zone formation in Starch Agar medium, highest amylase activity and non pathogenic characters. Thus, it was selected and used in further analysis. BAP6 isolate grew well in 2% Corn Flour Broth medium with the highest cell number, 2.2 x 10^8 CFU/ml at 24 h incubation and the highest amylase activity, 0.0201 Unit at 12 h incubation.

Key words: Amylase, amylolytic bacteria, corn, quail

INTRODUCTION

In recent years the application of enzymes as biocatalyst in industries has increased related to its special reaction in substrate (Mannervik *et al*., 2009). Amylase is one of important enzyme in industry which is used to breakdown amylum into simpler molecules like glucose or maltose. Three kinds of amylase were divided by Ekunsaumi (2002) based on their ability to breakdown amylum. They were alpha amylase, beta amylase and amyloglucosidae. Amylase has wide potential application in a number of industrial processes such as in the food, paper, textiles, fruit juices, sweeteners and spot remover in dry cleaning (Qader *et al*., 2006).

Amylase can be found in animals, plants and microorganisms. Amylase produced by microorganisms have some advantages as compared with the other sources.

Microorganisms are able to produce enzyme effectively in narrow space and takes only shorter time for production. The microorganisms are among others *Pseudomonas* sp., *Streptomyces* sp., *Aspergillus* sp., *Penicillium* sp., *Mucor* sp. (Suhartono, 1989), *Rhizopus* sp. (Omemu *et al*., 2005), *Bacillus* sp. (Qader *et al*., 2006) and *Bifidobacterium* (Reyed, 2007).

This research used amylolytic bacteria isolated from quail's (*Coturnix japonica*) digestive tracts. Corn with it high energy content related to its high amylum is the most proportion and important feedstuff in quail's ration. Amilolytic microorganisms are able to digest amylum by producing an extracellular amylase which breakdown amylum into simpler molecules (Ray, 2001) which is important to increase its absorbtion from the digestive tract. Thus, it is important to learn more about corn as medium for amylolytic bacteria growth and its amylase activity. The present study deals with the isolation of amylolytic bacteria from quail's digestive tract and the effect of corn flour as growing medium on growth or cells number and amylase activity of the bacteria.

MATERIALS AND METHODS

Isolation and preservation of amylolytic bacteria: Amylolytic bacteria used in this study were isolated from quail's (*Coturnix japonica*) digestive tract. Five gram of quail manure was added to 45 ml of NaCl 0.85%. Serial dilutions were followed by plating 0.1 ml sample on Starch Agar medium and incubated at 37°C for 48 h (Kar and Gosh, 2008).

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Pure isolates were maintained on Starch Agar slant and stored at 4°C for further studies (Qader *et al*., 2006). Isolates were maintained in glycerol stocks and stored at -20°C and -70°C for longer period of preservation (Van Dyke Laboratorium, 2008).

Screening of isolates: Screening of bacteria were done by isolating clear zone which was formed on Starch Agar medium and amylase activity was measured by DNS (dinitrosalicyclic acid) method. Fresh isolates were transferred into Starch Agar medium then incubated at 37°C for 48 h. Plates with bacteria colonies were then flooded with 0.1% iodine solution to form homogenous blue colour media. If the isolates were amylolytic bacteria, then they would hydrolyzed the starch present in their surrounding medium and then a clear zone formation would took place (Mishra and Behera, 2008). The cultured cell in Starch Broth medium (24 h and 48 h incubation) were removed by centrifugation at 4000 rpm for 15 min. Supernatant was used as the enzyme source. Amylase activity assay of the enzymes was done by using mixture consisting one ml substrate solution (1% soluble starch in 50 mM phosphate buffer pH 7) and 0.1 ml enzyme solution. The mixture was then incubated for 10 min at 37°C. The reaction in the mixture was stopped by adding 2 ml of DNS reagent (Bailey *et al*., 1992) then heated at 100°C for 10 min and cooled (Ajayi and Fagade, 2003). Optical density of each sample was measured at 540 nm in a spectrophotometer (Shimadzu). Enzyme activity was expressed in unit (1 unit = amount of glucose (in mole) released by enzyme under the assay condition).

Identification of isolates: Identification of isolates was performed by pathogenicity test in Blood Agar medium, morphological observation, Gram staining, endospore staining, catalase and oxidase test.

Growth curve and amylase activity in corn flour medium: Isolate was inoculated in 2% Corn Flour medium and incubated at 37°C overnight. Culture of isolate was transferred into 45 ml Corn Flour medium, then 15 ml from this overnight culture was transferred into 150 ml sterile Corn Flour medium. These media were incubated at 37°C in continuous shaking incubator (Kuhner) at 120 rpm. Growth curve was established by Total Plate Count (TPC) method. Growth curve and culture samples for amylase activity assay were measured every six hours.

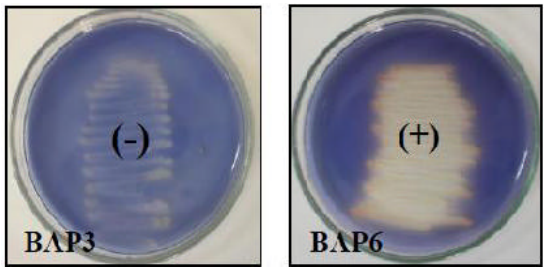

Fig. 1: Starch Agar plate with amylase activity showing clear zone formation (+) and no amylase activity showing no clear zone formation (-)

RESULTS AND DISCUSSION

Isolates of bacteria from quail's (*Coturnix japonica*) digestive tracts: Six isolates were obtained from quail's digestive tracts named as BAP1, BAP2, BAP3, BAP4, BAP5 and BAP6. Each isolate had different morphological colony (Table 1).

The ability of isolates in producing extracellular amylase: Extracellular amylase activity of isolates in hydrolyzing amylum was shown by clear zone formation in Starch Agar medium (Fig. 1). Only isolates BAP2 and BAP6 performed clear zone formation on Starch Agar medium. Melliawati *et al*. (2006) explained that bacteria produce amylase better in semisolid medium than in solid medium because cells of bacteria grow better in semisolid medium than in solid medium. No clear zone formation can caused by bacterial growth not in optimum condition.

Amylase activity assay showed that all of bacteria had amylase activity in Starch Broth medium (Table 2). The highest amylase activity at 24 h incubation was shown by two isolates, BAP2 and BAP4 and those at 48 h incubation was shown by four isolates BAP1, BAP3, BAP5 and BAP6. Based on those data, isolates BAP2 and BAP6 were then chosen for further studies because of their highest intensity of clear zone formation in Starch Agar medium and highest amylase activity in Starch Broth.

Characters of isolate: It was important to know whether the isolates were pathogen or not by pathogenicity test on Blood Agar medium (Fig. 2). Inoculation of bacteria on Blood Agar will perform three kinds of hemolysis, they are alpha, beta and gamma hemolysis. Alpha hemolysis

Table 1: Morphological colonies of isolates

Name of isolates	Colonies	Edge of colonies	Elevation	Inside structure of colonies	Colour	Diameter of colonies
BAP1	Circular	Entire	Convex	Translucent	Transparent	0.15 cm
BAP2	Circular	Entire	Convex	Opaque	Yellow	0.15 cm
BAP3	Circular	Entire	Low convex	Opaque	Dark white	0.20 cm
BAP4	Circular	Entire	Low convex	Opaque	Orange	0.35 cm
BAP5	Circular	Entire	Low convex	Opaque	White	0.25 cm
BAP6	Circular	Entire	Low convex	Opaque	Pink	0.15 cm

Table 2: Amylase activity of isolates

Name of isolates	Clear zone	Amylase activity (Unit) 24 h	48 h
BAP1	-	0.0348±0.0016	0.0247±0.0027
BAP2	+	0.0421±0.0015	0.0095±0.0022
BAP3	-	0.0256±0.0015	0.0247±0.0000
BAP4	-	0.0421±0.0015	0.0064±0.0015
BAP5	-	0.0046±0.0016	0.0211±0.0016
BAP6	+	0.0120±0.0000	0.0247±0.0027

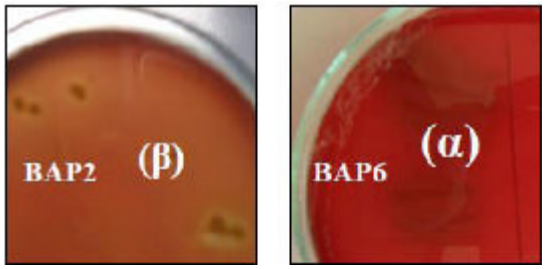

Fig. 2: Blood Agar plate showing hemolysis activity: (β) beta hemolysis on isolate BAP2 and (α) alpha hemolysis on isolate BAP6

(α-hemolysis) means that the bacterial enzymes only partially break down the blood cells which is indicated by yellowish/greenish/brownish discoloration of the media around bacterial colonies, indicating incomplete hemolysis. Beta hemolysis means that the bacterial enzymes completely break down the blood cells, indicated by clear zone in the media around bacterial colonies. Gamma hemolysis is essentially no hemolysis at all, indicated by no colour change in the medium. Blood Agar is useful to detect the presence of *Streptococcus* that causes beta hemolysis on Blood Agar. The major human pathogen in this group is *Streptococcus pyogenes*, the causative agent of strep throat. Normal throat flora will exhibit alpha or gamma hemolysis (Neogen Corporation, 2004).

Blood Agar is also used to detect pathogenic bacteria in poultry. Pathogenic bacteria, for examples *Gallibacterium* (Campogarrido *et al*., 2003) and *E. coli* (Murthy *et al*., 2008) will exhibit beta hemolysis on Blood Agar.

Figure 2 showed different kinds of hemolysis on isolate BAP2 and BAP6. Isolate BAP2 showed pathogenic character or beta hemolysis on Blood Agar, which meant that bacterial enzymes completely broke down the blood cells in the medium.

Isolate BAP6 was selected for further analysis because of its widest clear zone formation in Starch Agar medium and its highest amylase activity also non pathogenic character. Pathogenic characters of isolate BAP6 was shown in Table 3.

Isolate BAP6 is Gram negative coccus bacteria with 2 µm in diameter of cell. Gram staining and endospore

Table 3: Pathogenic characters of isolate BAP6

Characters	Results
Morphological colony	Colony *circular*, edge of colony *entire*, elevation *low convex*, inside structure of colony *opaque*, pink in colour and diameter of colony 0.15 cm
Clear zone	+
Hemolysis on Blood Agar	Alpha (α-hemolysis)
Gram staining	Negative
Cell form	Coccus
Diameter of cell	2 µm
Endospore staining	-
Catalase test	+
Oxidase test	-

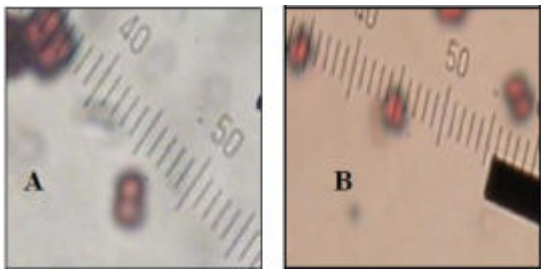

Fig. 3: Gram staining (A) and endospore staining (B) of isolate BAP 6 (1000x magnification)

staining were shown on Fig. 3. Gram negative bacteria has thin peptidoglycan and an outer membrane. Alcohol will dissolve outer membrane then safranin will counterstain bacteria resulting red colour cell (Linardakis, 1998). Isolate BAP6 did not perform endospore.

Catalase test showed positive reaction, which meant that isolate BAP6 was able to produce catalase enzymes. The presence of O_2 can produce H_2O_2 (hydrogen peroxide) which is toxic for microorganism's cell. Obligate aerob and facultative anaerob microorganisms produce catalase enzymes (Todar, 2000). Oxidase test showed negative reaction as shown by no change in colour of test kit. It meant that there was no sitochrome C in isolate BAP6 (Yap *et al*., 1999).

Observation of corn flour concentration: It is important to measure corn flour concentration as the best indicator for the growth of isolate. Measurement of cells was carried out on overnight cultured medium at 37°C and pH 6.8. Data in Table 4 showed that better bacteria cells growth was resulted in 2% corn flour concentration as compared to those in 1% corn flour concentration.

To facilitate extracellular amylase of bacteria degrade amylum, the medium (the corn) must be in flour form. Suhartono (1989) stated that smaller molecules of medium is well penetrated by extracellular enzyme than the bigger ones.

Table 4: Growth of isolate BAP6 in different corn flour concentration

Corn flour concentration	Number of cells (cells/ml)
1%	$2.22 \times 10^7 \pm 0.007$
2%	$3.52 \times 10^7 \pm 0.007$

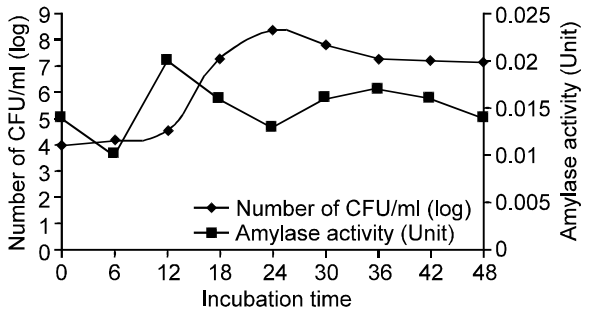

Fig. 4: Growth curve and amylase activity of isolate BAP6 in 2% Corn Flour broth

Growth and amylase activity of isolate in 2% corn flour broth: Growth curve and amylase activity of isolate BAP6 were shown in Fig. 4. Lag phase was reached in 12 h incubation time. This long period of lag phase was due to isolate BAP6 was transferred from Starch Agar medium to 2% Corn Flour broth. Brown (2007) presented that slowly growth in lag phase was related to bacterial adaptation from one medium to the other medium.

Highest cell number of isolate was shown by 24 h incubation time; 2.2×10^8 CFU/ml while highest amylase activity; 0.0201 unit was shown by 12 h incubation time. Cell number has negative correlation with amylase activity. Increasing cells number of isolate followed by decreasing amylase activity. Previous research (Ikram-Ul-Haq *et al*., 2009) explained that amylase activity was affected by cultivation methods, substrates (medium) ingredient or toxic accumulation in culture medium.

Amylase activity also related to the microorganisms characters. Decreasing amylase activity was also caused by increasing other enzymes activity. Microorganisms usually degrade carbohydrate first then they will degrade the other composition of nutrition (Mohney, 2006).

The disadvantage of corn as amylase production medium was high chemical ingredient in corn. It is complicated to know the chemical which able to induce amylase activity (Ajayi and Fagade, 2006).

In conclusion, this study result in six isolate of bacteria named BAP1, BAP2, BAP3, BAP4, BAP5 and BAP6. Corn flour medium in 2% concentration showed increasing cell numbers of isolate followed by decreasing amylase activity. Highest cell number of isolate showed by 24 h incubation time 2.2×10^8 CFU/ml and highest amylase activity 0.0201 Unit showed at 12 h incubation time.

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