The amylase production by Actinobacteria isolated from rumen fluid

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Abstract. Amylolytic actinobacteria were isolated from the rumen fluid of ruminants. Eighteen isolates actinobacteria were obtained and the generation of actinobacteria mutants using chemical agent Sodium Azide. The actinobacteria wild type and mutants were then screened for amylase activity using starch agar plate assay. Ten isolates showed the ability to produce amylase enzymes. Submerged fermentation (SmF) was used for microbial production of amylase. It was found that maximum amylase activity (2.53 U/mL) was produced by actinobacteria isolate R-9 M, and 2.43 U/mL was produced by isolate R-2 WT using pollard as a substrate in Submerged fermentation. This preliminary study could provide base information for the discovery of novel actinobacteria from natural resources such as rumen fluid for the production of amylase, which will be used for multipurpose.

1. Introduction
Actinobacteria are high-GC content, gram-positive and filamentous bacteria which are found freely or saprophytically in different habitats. They are widespread in terrestrial and aquatic habitats. They are widely distributed in terrestrial and aquatic habitats. They include important genera such as Micromonospora, Nocardia and Streptomyces whose large genomes enable them to produce some types of secondary metabolites, antibiotics, industrial enzymes, antitumor materials, nutritional metabolites and pesticides [1]. There have been various reports of starch degrading microorganisms from various sources with their amount of amylase activity [2].
Actinobacteria in the rumen gut of Ruminantia have not been investigated extensively. The rumen is an anaerobic “bioreactor”, containing a multitude of microorganisms that efficiently converts complex organic compounds. Therefore, exploring the rumen biodiversity may yield dedicated microorganisms capable of producing certain useful enzymes, such as amylase, which is an important enzyme that converts and digests feed in the rumen.

Amylolytic enzymes are of great significance in biotechnology, which is obtained from various plant sources, animals and microorganisms. Bacterial α-amylases are important and widely used enzymes. The short growth period of microbial sources made them meet industrial demands. Microbial amylase almost completely replaces chemical hydrolysis in the starch production industry, and this enzyme has wide applications in the fermentation, food and paper industries [3]. Besides their use in starch saccharification, they are also in the food, baking, brewing, detergent, textile, paper and distilling industries [4]. The significant advantages of using microorganisms for the production of amylases are the economical bulk production capacity of microbes and their easier manipulation to obtain enzymes with desired characteristics.

α-Amylases (endo-1,4-α-D-glucan glucohydrolase EC 3.2.1.1) are endo-extracellular enzymes that randomly cleave the 1,4-α-D-glucosidic linkage in a linear amylase chain of adjacent glucose units. Amylase has a wide distribution in the living system and has particular substrates [5]. Amylases are categorized into endoamylases and exoamylases. Starch molecules are hydrolyzed by endoamylases in an unknown manner, thereby causing oligosaccharide formation in a branched or linear manner.

The most important bacterial genera that produce amylase are Bacillus, Streptomyces, Micrococcus, Pseudomonas, Arthrobacter, Escherichia, Proteus and Serratia [6, 7]. Rumen microorganisms have a diverse range of enzymatic activity and capable of catalyzing various biochemical reactions with new enzymes such as cellulase, amylase, lipase and protease. Actinobacteria are one of the most investigated groups because they constitute a potential source of biotechnologically interesting substances. In this study, we first report the production of α-amylase secreted by actinobacteria that were isolated from rumen fluid of ruminant.

2. Material and Methods
2.1. Sample collection
Rumen fluid from a fistulated cattle was collected from Cibinong, West Java Indonesia

2.2. Isolation of the actinobacteria
Isolation of actinobacteria was done by serial dilution method, and dilutions were made up to 10^-6. Different dilutions were placed on Humic acid-Vitamin Agar (HVA) medium and kept for incubation at 30°C for 7 days. Actinobacterial cultures were observed after incubation and different cultures were selected, which differed in their shape, structure, elevation etc.

2.3. Mutation of actinobacteria through chemical agents using Sodium Azide [8]
The α-amylases productivity of actinobacteria can be efficiently increased through genetic manipulation [9]. The present investigation dealt with the improvement of α-amylases productivity of actinobacteria through chemical agent Sodium Azide mutagenesis. Each pure colony was streaked on a Starch Yeast Peptone plate containing various concentrations (10, 50 and 100 ppm) of sodium azide,
incubated at 28ºC for 5-6 days. Based on complete inhibition of actinobacteria growth, three categories are assigned as (a) Very sensitive (10 ppm lethal concentration), (b) Moderately sensitive (50 ppm lethal concentration), and (c) Tolerant (100 lethal concentration) ppm\[8\]. The colonies that have a different morphology from wild species are considered mutants.

2.4. Screening of amylase producing bacteria

All the 18 actinobacteria isolate wild type, and mutants were observed for amylase production by streaking the bacterial isolate on Starch Yeast Extract agar media, which have soluble starch as the carbon source. After incubation, the plates were flooded with 1% iodine solution and kept for 1 minute until the media got coloured in violet. The isolates showed the wider degrading zone around the bacterial colony, so these were chosen for further study because of its better starch degrading capability indicating amylase produced by actinobacteria.

2.5. Identification of actinobacteria

The molecular approach has been followed for the identification of the strains. Actinobacteria colonies were characterized based on 16S rRNA gene similarity with the closest species of actinobacteria on the database. Preparation of template DNA, PCR amplification and sequencing of 16S rDNA. Genomic DNA for sequencing 16S rDNA and G+C content was prepared according to the method of [10]. 16S rDNA gene was amplified by PCR using a pair of primers 9F (forward: 5’-GAGTTTTGATCCTGGCTCAG-3’ position 9-27) and 1541R (Reverse: 5’-AAGGAGGTGATCCAGGCC-3’ position 1541-1525). The 16S rDNA gene sequences obtained were used for BLAST analysis with the NCBI GeneBank database and aligned using Clustal W (multiple alignment software programmes). MEGA 7 version was used for phylogenetic tree construction and Distance matrix generation [11, 12].

2.6. Enzyme production medium

Production medium contained (g/l) Pollard 5 g, Soluble starch 5 g, peptone 2 g, and Yeast extract 2 grams. One hundred ml of medium was taken in a 500 ml conical flask. The flasks are sterilized in an autoclave at 121 oC for 15 minutes and after cooling the flask was inoculated with overnight grown actinobacteria culture. The inoculated medium was incubated at 28 ºC in shaker incubator for 5 days. At the end of the cultivation period, culture media were centrifuged at 9000 rpm for 15 minutes to obtain a crude extract, which served as an enzyme source.

2.7. Crude enzyme extraction is sterilized in an autoclave at 121 oC for 15 minutes

After the incubation, it was centrifuged for 10 minutes, and the supernatant was collected, which is the crude enzyme. The crude enzyme was checked for enzyme activity by amylase assay.

2.8. Amylase assay

The activity of α-amylase was estimated by determining the amount of reducing sugar released from starch. Amylase assay was done by DNS method [13]. One hundred µl of the 1% starch solution was added to each tube and incubate for 5 minutes at room temperature to reach temperature equilibration. Perform the first add of the amylase solution 50 µl, 75 µl and 100 µl to the respective microtubes. The
mixture solution was mix with inversion and incubated at room temperature for 5 minutes. The reaction was stopped by adding 100 µl of DNS solution to each tube. Place tubes in the heating block and boil at 100 °C for 15 minutes. After boiling the solutions were cooled on ice for 10 minutes. After reaching the room temperature, add 900 µl of purified water to each tube and mix by inversion and absorbance was read at 540 nm using maltose as standard. One unit (U) of enzyme activity is defined as the amount of enzyme required for the liberation of 1µmol of reducing sugar as glucose per minute under assay condition.

3. Result and Discussion

3.1 Isolation and primary screening of amylase producing actinobacteria

In this study, actinobacteria were isolated from rumen gut fluid. Rumen fluid was serially diluted and isolated on Humic acid Vitamin Agar. A total of 18 isolates were obtained from the collected rumen fluid and coded as R-1 – R-18. Wild type actinomycetes were initially classified based on their sensitivity to sodium azide and then used to produce mutants through mutations using chemicals. Eighteen actinobacterial isolates from rumen fluid were grown on medium containing sodium azide concentrations of 10, 50 and 100 ppm, so were obtained in a very sensitive group, quite sensitive and tolerant of Sodium azide. (Table 1).

Table 1. Categories of the actinobacteria (total number of isolates=18) based on their sensitivity to sodium azide

| No Isolate | Wild (10 ppm) | Highly Sensitive (10 ppm) | Moderate (50 ppm) | Tolerant (100 ppm) |
|------------|--------------|------------------------|------------------|--------------------|
| R-1        | +            | +                      | -                | -                  |
| R-2        | ++           | ++                     | +                | -                  |
| R-3        | +            | -                      | -                | -                  |
| R-4        | +++          | ++                     | ++               | -                  |
| R-5        | ++           | ++                     | +                | -                  |
| R-6        | +            | -                      | -                | -                  |
| R-7        | +++          | ++                     | +                | +                  |
| R-8        | +            | -                      | -                | -                  |
| R-9        | +++          | +++                    | +++              | +                  |
| R-10       | +++          | ++                     | ++               | +                  |
| R-11       | +++          | ++                     | ++               | +                  |
| R-12       | ++           | +                      | -                | -                  |
| R-13       | +++          | +++                    | ++               | +                  |
| R-14       | +++          | +++                    | +                | +                  |
| R-15       | +++          | +                      | +                | -                  |
| R-16       | +++          | +++                    | +++              | +                  |
| R-17       | +++          | ++                     | +                | -                  |
| R-18       | +++          | +++                    | +                | +                  |

← no growth, + = adequate growth, ++ = good growth, +++= abundant
Of the total 18 actinomycete isolates, four (27.7%) were highly sensitive, five (22.2%) were moderately sensitive, and eight (44.4%) were tolerant [8].

From the sensitive and tolerant actinobacteria, twenty-two isolates wild type, and mutants of rumen actinobacteria were characterized for their potential in production amylase enzyme, based on their starch degrading ability using Starch Iodine Plate method [14].

![Figure 1. Primary screening of amylase producing actinobacteria using Starch agar medium, A= R-2 WT and R-2 M, B= R-9 WT and R-9 M, C= R-14 WT and R-14 M.](image)

Primary screening for $\alpha$-Amylase was performed by inoculating the strains on Agar media followed by flooding with Iodine solution, stay for 5 minutes, amylase producing strains were identified by the formation of the zone of clearance against the blue colour background [15]. The ability of rumen actinobacteria isolates to degrade starch can be seen from the diameter of the formation of a clear zone. From the results of diameter of clear zones, isolates of rumen Actinobacteria which had the highest diameter clear zone values were isolates R-2 WT, R-9 WT, R-9 M, R-2 M, and R-14M with a diameter clear zone values (mm) of 38.84 ± 0.69, 26.65 ± 0.03, 26.30 ± 0.58, 25.98 ± 0.70 and 20.57 ± 1.47 (Figure 1 and Table 2) respectively. In starch degradation test, five isolates showed Loss of function (LOF) that actinobacteria wild-type formed larger halo than their mutants, and six isolates showed Gain of Function (GOF) that their mutant formed larger halo-forming zones than that obtained by the parent strain. For this wild-type and mutants, quantitative determination of their amylase productivity was tested.

Table 2. Isolates and their clear zone on starch agar plates during primary screening

| Isolates | Clear zone (mm) | Isolates | Clear zone (mm) |
|----------|----------------|----------|----------------|
| R-2 WT   | 38.84 ± 0.69   | R-2 M    | 25.98 ± 0.70   |
| R-4 WT   | 13.87 ± 1.18   | R-4 M    | 22.30 ± 0.60   |
| R-7 WT   | 5.46 ± 0.02    | R-7 M    | 12.53 ± 0.03   |
| R-9 WT   | 26.65 ± 0.03   | R-9 M    | 26.30 ± 0.58   |
| R-10 WT  | 5.88 ± 0.40    | R-10 M   | 4.36 ± 0.41    |
| Isolate | WT Activity | Mutant Activity |
|---------|-------------|----------------|
| R-11    | 11.51 ± 0.01 | R-11 M         |
| R-13    | 4.88 ± 0.35  | R-13 M         |
| R-14    | 18.21 ± 0.31 | R-14 M         |
| R-15    | 17.49 ± 0.14 | R-15 M         |
| R-16    | 7.16 ± 1.04  | R-16 M         |
| R-18    | 4.51 ± 0.55  | R-18 M         |
| R-11    | 11.51 ± 0.01 | R-11 M         |
| R-13    | 4.88 ± 0.35  | R-13 M         |
| R-14    | 18.21 ± 0.31 | R-14 M         |
| R-15    | 17.49 ± 0.14 | R-15 M         |
| R-16    | 7.16 ± 1.04  | R-16 M         |
| R-18    | 4.51 ± 0.55  | R-18 M         |

**Figure 2.** Amylase activity of different actinobacterial isolates on Submerged fermentation medium containing pollard as substrate.

The range of amylase production by selected wild type (parents) and mutants was 0.04 to 2.45 U/mL (Figure 2). The highest amylase productivity was obtained by the mutant coded R-9 M (2.53 U/mL). Four mutants designed as R-4 M, R-7 M, R-9 M and R-16 M had higher amylase activity than that of the parent strain. Amylase activity of the mutants in the Submerged fermentation medium containing pollard broth was 4.88-, 11.34-, 1.07- and 2.03-fold of the parent strain, respectively. The enzyme activity of the mutant (R-9 M) is high and calculated to be 2.53 U/mL. However, amylase activity from other mutants is low compared to the original strain. Hence, Sodium azide mutation is found to have negative feedback on amylase production in the rumen actinobacteria strain [16].
In this study, the agro-waste pollard was used as a substrate of α-amylase production, some different substrates like wheat bran, maize bran, corn bran, millet bran, cassava peel powder, cottonseed oil cake, coconut oil cake, sesame oil cake, groundnut oil cake and so on, where used as a substrate for α-Amylase production [17]. In this study with pollard as substrates used, it was found that the maximum amylase activity (2.53 U/mL) was measured by strain R-9 M using submerged fermentation technique. Senthilkumar et al. [18] reported that when cassava was used as a substrate; the amylolytic activity was found to be 2.9 U/ml at 30°C. The highest activity in *Streptomyces clavifer* was observed at 3.78 U/mL [19].

From seven genomics DNA that was isolated, only five were successfully sequenced based on 16S rRNA gene and showed that all five rumen actinobacteria found to belong to the genus Streptomyces. Phylogenetic analyses revealed that strains R-2, R-4, R-7, R-14 and R-16 are related to the members of the family Streptomycetaceae. However, the strains did not form a reliable clade with the closest species members of the family Streptomycetaceae (Figure 3). It is possibly indicating the five isolates as new taxon within the Streptomycetaceae family.

![Figure 3. Phylogram derived from 16S rRNA gene sequences of 5 isolates of rumen actinobacteria and their related strain, showing the relationship with type strain in a cluster node of Streptomyces. Bootstrap values based on 1000 replications are shown at branch nodes; 16S rRNA gene of the genus Actinomadura was used as the out group, located at the basal phylogram.](image-url)
Phylogenetic analysis revealed that isolate R-4, R-7 and R-16 forms a distinct branch with the closest species *S. matensis* NBRC 12889. Isolate R-2 and R-14 appears to represent a distinct taxon related with the closest species *S. cavourensis* NRRL 2740.

4. **Conclusion**

In conclusion, the rumen gut actinobacteria serve as the potential bioactive for the α-Amylase enzyme. The study on enhancement of amylase production from Actinobacteria strain improvement through mutation by chemical agents showed that five isolates Loss of function (LOF), which actinobacteria wild-type formed larger halo than their mutants, and six isolates showed Gain of Function (GOF) that their mutant formed larger halo-forming zones than that obtained by the parent strain in starch degradation test. Sodium azide mutation seems to have negative feedback on amylase production in the rumen actinobacteria strain. Actinobacterial communities at the specific habitats in the rumen fluid ecosystem are potential as a source for isolation the new taxon of actinobacteria. The present results show that actinobacteria are present and alive in the rumen gut. The distribution of actinobacteria into rumen fluid possibly carried by forage feeds from ruminants.

5. ** References**

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