RESEARCH ARTICLE

Screening of presumptive amylase-producing bacillus bacteria isolated from soil containing dairy food waste

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Abstract:

Soil microorganisms can produce amylase, especially the *Bacillus* strains bacteria. Amylase-producing bacteria can be obtained from soil containing properly composted dairy food waste. This study aims to analyse amylase-producing bacteria obtained from soil contaminated with dairy food waste. The soil sample was collected in three different settings, which were A: normal soil without food waste, B: soil with yoghurt-based dairy food waste and C: soil with mixed dairy food waste. The isolation of bacteria was done and screened for α-amylase-producing bacteria using a starch hydrolysis test. Based on the result, a total of 17 gram-positive isolates were identified. They were classified into spore-forming and catalase-positive bacteria. Nevertheless, seven out of 17 isolates showed a positive for amylase production. Presumptive identification of the soil sample showed that soil A contains amylase-producing bacteria, presumably non-*Bacillus* spp. In contrast, soil samples B and C, which after being contaminated with dairy food waste, contain amylase-producing bacteria, most likely *Bacillus* spp. As exhibited by the characteristics of isolates B1, B3, B7, and C3. In conclusion, the amylase-producing bacteria from soil contaminated with dairy food waste were identified and classified.

Keywords: amylase, *Bacillus* spp., dairy food waste, soil

1. INTRODUCTION

Amylases are essential enzymes that have various applications in many industries nowadays. They can be obtained from multiple sources, such as plants, animals and even microorganisms. Usually, the microbial alpha-amylase (α-amylase) such as fungi, yeast, and bacteria possesses many advantages in the industrial demands where they have high capability to fulfil the commercial need of the industry (de Souza & de Oliveira Magalhães, 2010). The wide diversity of α-amylase attracts the attention of many researchers in exploiting their energy and carbon sources for physiological and biotechnological applications (Ju et al., 2019; Saini et al., 2017). Additionally, based on previous studies, the microorganisms that are more capable of producing α-amylase for industrial application are from *Bacillus* strains bacteria such as *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus subtilis*, and *Bacillus stearothermophilus*, including the actinomycetes, and fungi (Far et al., 2020).

Foods manufactured predominantly from milk are known as dairy products. Examples include ice cream, yoghurt, cheese, condensed milk, dried milk, cream cheese, and butter. The demands for dairy products in Malaysia continue to increase as the changes in Malaysian diets from starch-based products are influenced by the rising concern about food quality, safety and nutritional food content (Boniface & Umberger, 2012). In turn, dairy products are listed as a massive contributor to food waste as these products can easily get spoilt. Furthermore, the products do not have a long shelf life and can often be discarded because they are not correctly stored at the right temperature and are not used within the expiration date. According to Hickey et al. (2015), the most common microorganism of dairy products is the lactic acid bacteria (LAB). It is considered an essential starter culture in fermented dairy products. Examples of LAB associated with dairy products are *Streptococcus*, *Lactococcus*, Lactobacillus, *Bifidobacteria*, *Enterococcus* and *Pediococci* (Hickey et al., 2015). Some LAB, such as *Lactobacillus amylovorus*, *Lactobacillus plantarum*, *Lactobacillus manihotivorans*, and *Lactobacillus fermentum* produce amylase enzymes because of their exhibiting amylolytic activity (Padmavathi et al., 2018). A previous study found that most α-amylases isolated from lactic acid bacteria show weak thermostability compared to the *Bacillus* strain. However, α-amylases from a strain of Lactobacillus fermentum show high thermostability, which is considered the upper hand because they are generally regarded as safe (GRAS) microbe (Fossi et al., 2016).

In addition, microorganisms in soil are the most accessible source to collect the organism for isolating the amylase-producing bacteria as they live in their natural pH and

temperature environment. Plenty of nutrients will become food sources for the soil microbes as they receive them from dead plant residues or the plant nutrients, untreated sewage, improper waste management such as animal waste, industrial sources, and some landfills. Food wastes are one of the primary nutrient sources for soil microorganisms. It is an essential commodity that contributes to most organic waste generated worldwide (Sharma et al., 2020). Since the soil is a natural source of microbial α-amylase producers as it can harbour numerous sources of microorganisms, thus, isolation of amylase-producing bacteria from the soil will be less tedious and the most convenient way to obtain prominent colonies of the bacteria for further studies. Therefore, this study is carried out to identify presumptive amylase-producing bacteria from soil contaminated with dairy products.

2. MATERIALS AND METHODS

2.1 Soil Sample Preparation

Soil compost was prepared a few months earlier prior to the test. Since dairy products were well-known for their high moisture and fat content, it was best to mix them with dry fibrous materials. Dry leaves, shredded paper and black soil were added into the garden pot until up three-fifths whole and mixed the rest with the dairy waste products. It was preferable to use a small number of dairy products with fibrous materials to help counteract the compost's wetness and lack of texture (Anthony, 2019). The soil compost was turned regularly to aerate it, incorporating oxygen into the soil and generally speeding up the composting process. An anaerobic decomposition was undesirable since the dairy product was highly prone to lousy odour. Besides, the compost pile was placed in a covered and dry place as dairy food waste tends to yield a high amount of leachate. The leachate production was routinely cleaned. Hence, reducing the attraction of pests and ensuring the compost's safety.

2.2 Collection of Soil Samples

There were three types of soil selected for this study. The first was normal soil which does not contain any food waste. Second, soil contaminated with yoghurt-based dairy food waste and third, soil contaminated with mixed dairy food waste products. They were labelled as Soil A, Soil B, and Soil C, respectively.

The soil sample was collected randomly at a depth of 5 cm to 10 cm after the top layer to avoid contamination with other surfaces using a clean spatula in a small beaker. Their physical-chemical properties, such as pH and temperature, were recorded for the quantitative result. Soil pH was achieved by mixing soil and distilled water with a ratio of 1:1 and measured using a pH-Meter 765 Calimatic (Knick).

2.3 Isolation of bacteria from soil sample (primary culture)

The isolation of microorganisms from soil samples was done through a serial dilution technique and cultured on a nutrient agar medium as suggested by Kannan et al. (2018) with a few modifications. First, five clean test tubes were prepared and labelled for each samples A, B, and C. Then, 0.4 g of each soil sample was suspended in 4 ml of sterile distilled water in each first test tube, respectively. Next, the soil suspension was mixed using a vortex mixer for 15 minutes, followed by incubation in a water bath at 85°C for 15 minutes of heat treatment. Then, the soil suspension was incubated at room temperature for 2 hours (Afzal-Javan & Mobini-Dehkordi, 2013).

A 5-fold serial dilution was done by transferring 1 ml of the prepared soil suspension into their new test tube set containing 4 ml of distilled water each. The process was repeated until the 4th dilution for every sample. Then, it was inoculated on nutrient agar and incubated at 37°C for 24 hours aerobically. After incubation, each bacteria colony was subcultured on nutrient agar to obtain a pure colony and maintained at 4°C for further analysis (Kannan et al., 2018).

2.4 Obtaining a pure culture of the bacterial isolates

The pure culture isolates were identified from a single colony by analysing the appearance of culture morphology. Other characteristics were examined through Gram staining, the presence of bacterial spore by endospore stain and catalase reactions.

2.5 Identification of amylase-producing bacteria

A starch hydrolysis test was carried out according to Luang-In et al. (2019) with some modifications for the identification of amylase-producing bacteria. First, gram-positive bacilli were selected and cultured on a starch agar in a straight line using a sterile loop. Then, the agar plate was incubated at 37°C for 48 hours. Following the incubation, the surface of the agar plate was flooded with iodine using a sterile dropper, and the excess iodine was poured off. A clear zone surrounding the line of bacterial growth, which indicates a positive result of amylase production, was observed within 30 seconds (Luang-In et al., 2019). Bacterial culture with positive amylase production was carefully collected and stored in glycerol stock kept at a temperature of -80°C.

3. RESULTS AND DISCUSSION

3.1. Collection of soil

The soil samples were collected from three different sites (A, B and C). They were labelled appropriately and evaluated for temperature and pH readings prior to sample testing. The data collected were tabulated in Table 1.

Table 1: Result for temperature and pH reading of soil samples from setting A, B, and C.

Sample	Sample type	Temperature (°C)	Soil pH
Soil A	Normal soil	25°C	6.89 (neutral)
Soil B	Soil contaminated with yoghurt-based waste	25°C	6.48 (Weak acidic)
Soil C	Soil contaminated with mixed dairy waste	25°C	6.38 (Weak acid)

3.2 Colony morphology and gram stain

The sample collected from every setting was gone through serial dilution and cultured on nutrient agar for bacteria isolation. After 24 hours of incubation, the single colony bacteria were selected for colony morphology and microscopic examination using a gram stain. In soil A, five unknown bacteria were obtained, and three of them were gram-positive. Soil B obtained 11 unknown bacteria, and 10 of them were gram-positive. Finally, soil C consists of six unknown bacteria whereby, four of which were gram-positive. Among all isolated bacteria, a total of 17 gram-positive bacilli were identified (Fig 1).

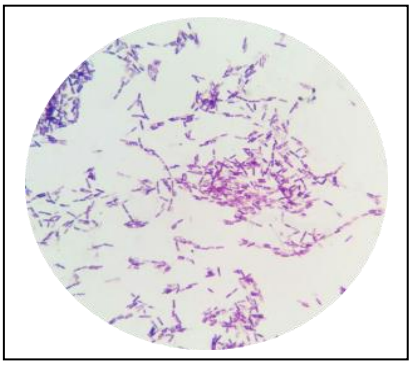

Fig 1: Gram-positive bacilli of the representative isolated bacteria (sample B3) observed with a light microscope (100X magnification).

3.2 Biochemical and screening test for amylase bacteria

All gram-positive bacterial isolates were further characterized using endospore stain and catalase test. Their results were tabulated in Table 2, whereas out of 17 bacteria isolates, seven of them were spore-forming bacteria. On the other hand, 11 isolated bacteria were recorded as catalase positive. Figure 2 showed the endospore staining result of an isolate which was spore-forming bacteria. Then, for screening for α-amylase bacteria, only seven isolates showed positive amylase production on the starch hydrolysis test such as in Figure 3. The clear zone surrounding the line of bacterial growth indicates a positive result of amylase production.

Table 2: Biochemical test and screening for amylase bacteria.

	Endospore stain	Catalase test	Starch Hydrolysis test
A1	Non-spore-forming	Negative	Positive
A2	Non-spore-forming	Negative	Negative
A3	Non-spore-forming	Negative	Positive
B1	Spore-forming	Positive	Positive
B2	Spore-forming	Positive	Negative
B3	Spore-forming	Positive	Positive
B4	Non-spore-forming	Positive	Negative
B5	Non-spore-forming	Positive	Positive
B6	Non-spore-forming	Positive	Negative
B7	Spore-forming	Positive	Positive
B8	Spore-forming	Negative	Negative
B9	Non-spore-forming	Negative	Negative
B10	Non-spore-forming	Positive	Negative
C1	Non-spore-forming	Positive	Negative
C2	Non-spore-forming	Negative	Negative
C3	Spore-forming	Positive	Positive
C4	Spore-forming	Positive	Negative

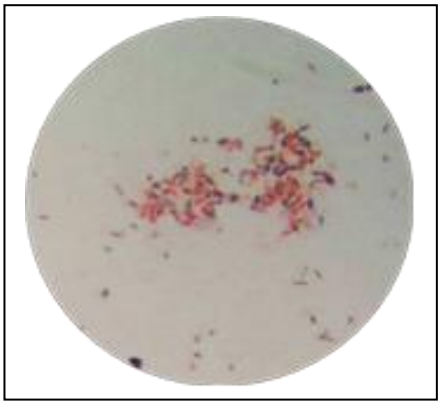

Fig 2: Endospore staining from a bacterial isolate. The vegetative cells are stained red, while the endospores are stained green (100X magnification)

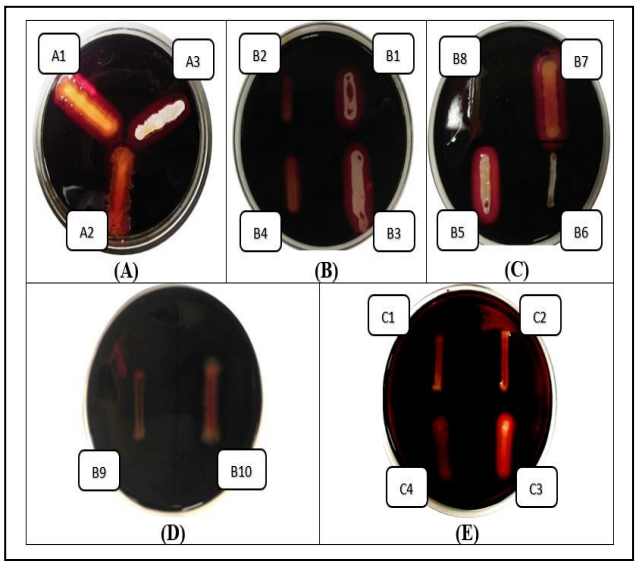

Figure 3**:** Starch hydrolysis test to screen presumptive amylase-producing bacteria. **(A)** Isolates from soil A showed positive for isolate A1, negative for isolate A2, and positive for isolate A3; **(B)** Isolates from soil B showed positive for isolate B1 and B3, negative for isolate B2, and B4; **(C)** Isolates from soil B showed positive for isolate B5 and B7, negative for isolate B6 and B8; **(D)** Isolates from soil B showed negative for isolate B9 and B10; **(E)** Isolates from soil C showed negative for isolate C1, C2 and C4; positive for isolate C3.

3.3 Discussion

In this study, the screening for the presumptive α-amylase-producing bacteria was obtained from soil contaminated with dairy food waste. Dairy products were specifically chosen for this study because not only do the demands for dairy products in Malaysia continue to elevate, but the possibility for them to be listed as one of the massive contributors to food waste were also high since these products can easily get spoilt (Boniface & Umberger, 2012). In addition, the soil is a natural source for microbial α-amylase producers such as *Bacillus* spp. due to their ability to adapt to various environmental conditions (Parvathi et al., 2009). Above all, amylases from microorganisms are preferred to be used in industrial applications due to their higher stability and ease of utilization compared to other amylases derived from plants and animals (de Souza & de Oliveira Magalhães, 2010).

The main objective of this study was to identify the amylase producer from the presumptive *Bacillus* strain isolated from the contaminated soil. Hence, only gram-positive bacilli were selected to proceed for identification after being tested with the gram stain procedure. Endospore stain and catalase test were performed to characterize the *Bacillus* spp. The endospore staining was helpful in differentiating gram-positive rods, as only *Bacillus* and *Clostridium* produce endospores (Reynolds et al., 2009). Therefore, the study proceeded with the catalase test to differentiate those aero-tolerant strains which are catalase negative for *Clostridium* and catalase positive for *Bacillus* (Reiner, 2010). Hence, according to the presumptive identification results, it was assumed that isolates B1, B3, B7, and C3 might belong to *Bacillus* spp. However, considering the physiology of *Bacillus* spp. are broad, an expansion in biochemical testing was needed to identify the species (Parvathi et al., 2009).

In addition, the bacteria isolates were screened for the production of amylase using a starch hydrolysis test. The results showed that a total of seven positive isolates had a different clear zone of hydrolysis when flooded with iodine. *Bacillus subtilis* ATCC6633 was supposed to be used as a positive control, while *Escherichia coli* ATCC25922 was a negative control. However, the test was not performed due to the unavailability of the mentioned bacterial strains. Based on a previous study, the larger clear zone of hydrolysis diameter and shorter time taken to decolourize the iodine solution indicate the higher amylase activities of the bacteria (Yassin et al., 2021). From this study, soil sample A does contain α-amylase-producing bacteria, presumably *non-Bacillus* spp. In contrast, soil samples B and C, which after being contaminated with dairy food waste, contain α-amylase-producing bacteria, presumably *Bacillus* spp. such as isolates B1, B3, B7, and C3.

4. CONCLUSION

In conclusion, the presumptive amylase-producing bacillus bacteria from soil contaminated with dairy food waste were identified. There are some characteristics that differentiated the amylase-producing bacterial isolates from normal soil with dairy foods contaminated soil which need further confirmation tests to be carried out. This study showed that dairy food waste could also be a potential source for microbial production of amylase as it is more economical and cost-effective in fulfilling industrial demands.

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