

## INVESTIGATION OF ALPHA AMYLASE ENZYME PRODUCTION OF *BACILLUS* SPP. EXTRACTED FROM EXTREME ENVIRONMENTS AND THEIR BIOTECHNOLOGICAL APPLICATIONS.

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### Abstract

Alpha-amylase is an industrially important enzyme that catalyses the breakdown of starch into simple sugars. This study aims to investigate the production of alpha-amylase from *Bacillus* spp. isolated from Mahapelessa hot spring and Ussangoda coast in Sri Lanka. Amylase production was done using solid-state fermentation. Two kitchen wastes (Banana and potato peels) were tested as substrates. Previously isolated and sequenced *Bacillus* spp. from Mahapelessa hot spring (three *Bacillus subtilis* spp. Mmb4, mmb11, mmb14 ) and Ussangoda coast (two *Bacillus subtilis* spp. US14-3, US3-2, *Bacillus altitudinis* US6-1, *Bacillus stratosphericus* US12-2) were retrieved on Luria-Bertani (LB) media. The selected species were subjected to amylase screening to identify the amylase-producing bacteria. Then, amylase-positive species were fermented in LB broth with the waste substrate for the extraction of amylase. Next, the fermentation medium was shaken with 20mM phosphate buffer (pH=7) at 100 rpm for 30 minutes at 4 °C. After centrifuging the shaken medium at 7000 rpm for 20 minutes, the supernatant was collected as the crude alpha-amylase enzyme. The microplate-based starch-iodine assay was used to quantify the activity of amylase. The absorbance was taken at 580nm. All the selected *Bacillus* spp. were amylase-positive except *Bacillus subtilis* spp. mmb14. Amylase activity was reported high on potato peel substrate for cultures from both sites. The highest amylase activity was reported in *Bacillus subtilis* spp. mmb4 on the potato peel (2.75 U/mL) substrate while showing the highest activity (0.84U/mL) on the banana peel substrate as well. Among the Ussangoda coast cultures, the highest amylase activity(1.00 U/mL) was reported in US14-3 (*Bacillus subtilis*) on potato peel. According to the obtained results, the isolate mmb4 (*Bacillus subtilis*) is able to produce alpha-amylase at a relatively high temperature. Also, the obtained results confirmed that potato peel is a good waste substrate for the natural alpha-amylase production from bacteria.

**Keywords:** Alpha-Amylase, *Bacillus* spp., Solid-State Fermentation, Thermophilic, Waste

## Extended Abstract

### 1. Introduction

Alpha-amylase is an enzyme that plays a vital role in numerous industries, including food, pharmaceuticals, detergent, textile, pulp and paper industries. Among different enzymes used in industries, alpha amylase is the most versatile enzyme due to the abundance of starch (1). Alpha-amylase catalyzes the hydrolysis of  $\alpha$ -1,4-linkages in starch molecules, resulting in simple sugars like glucose, maltose and maltodextrins. Alpha-amylases can be obtained from different living organisms such as plants, animals and microorganisms. Microorganisms are the most preferred organism for alpha-amylase production due to their easiness of handling, favourable growth conditions, availability and cheap nutrient requirements. Among different microorganisms, bacteria and fungi are mostly used to produce alpha amylases for industrial applications. According to the literature, the genus *Bacillus* has mainly been used to produce alpha amylases for commercial applications (2). *Bacillus* spp. has a substantial potential for use in different industries due to their favourable characteristics, including the ability to secrete enzymes and other proteins into the extracellular medium, short fermentation cycles, rapid growth rate, and safeness of handling.

Microorganisms that have the ability to grow and survive in extreme environments are known as extremophiles. These microorganisms are adapted at the molecular level to survive under such extreme conditions. Therefore, extremophiles can produce enzymes that can function under harsh conditions such as high temperatures, salinity, pH and pressure. Such enzymes are known as extremozymes. Extremozymes have attained a special interest in various industries due to their stability at high temperatures and wide range of pH, which are the predominant conditions in most industrial processes. Among different extremozymes, thermostable and halophilic alpha-amylases are one of the major industrial enzymes.

Both solid-state fermentation and submerged fermentation methods can be used to produce alpha-amylase using microorganisms. But solid-state fermentation is extensively used as it possesses numerous advantages such as low capital investment, lower energy requirement, simplicity, and better product recovery than the other methods. Different solid substrates can be used for the fermentation of alpha-amylase-producing microorganisms.

Synthetic alpha-amylase production is a costly process which requires expensive chemicals. But using food wastes, natural alpha-amylase can be produced at a lower cost under solid-state fermentation. The use of food waste as the substrate for industrially using alpha amylase also provides a solution to the global waste problem. This study used food wastes such as banana peels and potato peels as substrates, and the aim of the study is to investigate the low-cost production of industrially important alpha-amylase from extremophilic *Bacillus* spp. under solid-state fermentation using food waste.

### 2. Methodology

a. Culture retrieving.

The pure cultures of previously isolated and sequenced *Bacillus* spp. from Mahapelessa hot spring and Ussangoda coast, which were stored in the project culture collection, were retrieved on Luria-Bertani (LB) agar media. Two *Bacillus subtilis* species (US14-3 and US 3-2), *Bacillus altitudinis* (US6-1) and *Bacillus stratosphericus* (US12-2) were selected from the Ussangoda cultures while three *Bacillus subtilis* species (mmb4, mmb11 and mmb14) were selected from the Mahapelssa hot spring cultures. After retrieving the cultures, Gram's staining was performed for all the selected *Bacillus* spp.

b. Amylase screening test.

An amylase screening test was performed on starch-agar media. A loop full of freshly grown bacterial cultures was inoculated on starch-agar media. Then, the inoculated media plates were incubated at 44.5 °C (Mahapelessa cultures) and 37 °C (Ussangoda coast cultures) for 24 hours. After 24 hours of incubation, the starch-agar media plates were flooded with the Iodine solution.

c. Fermentation.

All the amylase-positive species were fermented for the extraction of alpha-amylase enzyme. To prepare the inoculum, 20 µl of freshly grown bacterial culture was inoculated into 50 ml Luria-Bertani broth media in a 250 ml Erlenmeyer flask. The inoculum was incubated at 44.5 °C (Mahapelessa hot spring cultures) and 37 °C (Ussangoda coast cultures) for 48 hours. After incubation, 5g of the pre-treated substrate (Banana peels and potato peels) was separately added into the inoculation medium. Then, this fermentation media was incubated at 44.5 °C (Mahapelessa hot spring cultures) and 37 °C (Ussangoda coast cultures) for another 48 hours.

d. Extraction of crude alpha-amylase enzyme.

After the completion of the incubation period, the fermentation medium was soaked with 20 mM phosphate buffer (pH=7.0). Then, it was shaken at 100 rpm for 30 minutes at 4 °C on an orbital shaker (ORBITEK). Next, the medium was centrifuged (Centrifuge 5430R, Eppendorf) at 7000 rpm for 20 minutes at 4 °C. The supernatant was collected as the crude alpha-amylase enzyme, and it was stored at -20 °C until further use.

e. Quantification of alpha-amylase activity.

The enzyme activity of extracted crude alpha-amylase was quantified using the microplate-based starch-iodine quantitative assay. Under this assay, 40 µl of starch solution (2g/l), 40 µl of 0.1M phosphate buffer (pH=7.0) and 40 µl of the crude enzyme were added into wells of a 96-well plate. To minimize the evaporation, the microplate was covered with a plastic mat, and it was incubated at 44.5 °C (Mahapelessa hot spring cultures) and 37 °C (Ussangoda coast cultures) for 30 minutes. After the incubation, 20 µl of 1M HCl was added to each of the sample-containing wells to stop the reaction. Then, 100 µl of iodine reagent was added to each well. Next, 150 µl of the iodine-treated samples were transferred to a flat bottom 96-well plate, and the absorbance was taken at 580 nm using a microplate reader (FLUOstar Omega, BMG LABTECH). The alpha-amylase activity was calculated using the below equation. Under this assay, it was assumed that one unit of enzyme is equivalent to the average loss of 1 mg of iodine-binding starch material per minute.

$$\text{Alpha-amylase activity (U/ml)} = \frac{A_{580} \text{ control} - A_{580} \text{ sample}}{\text{Volume of sample (ml)}}$$

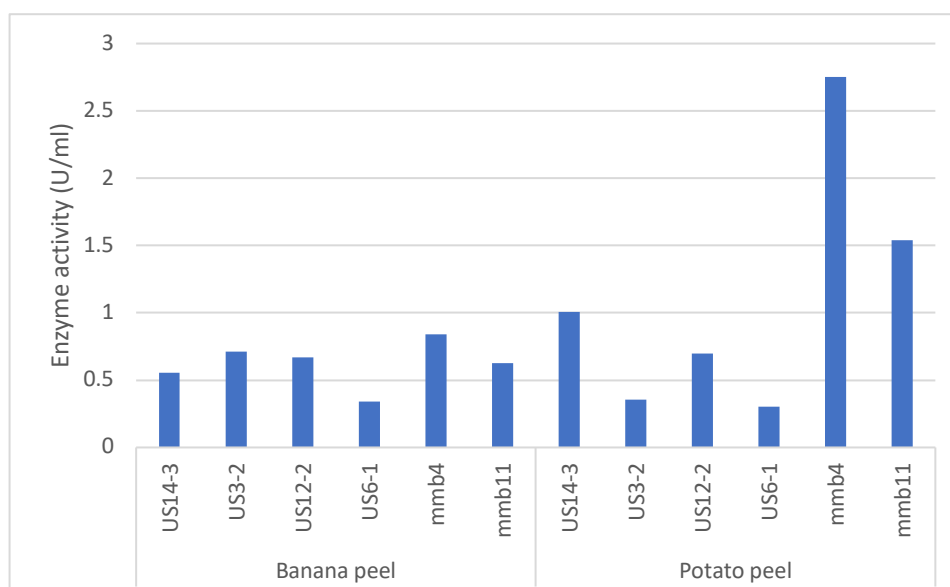
$$A_{580}/\text{mg starch} \times \text{assay time} \times \text{volume of the enzyme}$$

### 3. Results and Discussion

According to the amylase screening test results, all the selected *Bacillus* spp. were amylase positive except the *Bacillus subtilis* spp. mmb14 isolated from Mahapelessa hot spring.

**Table 01.** Alpha amylase activity of selected *Bacillus* spp. isolated from Mahapelessa hot spring and Ussangoda coast.

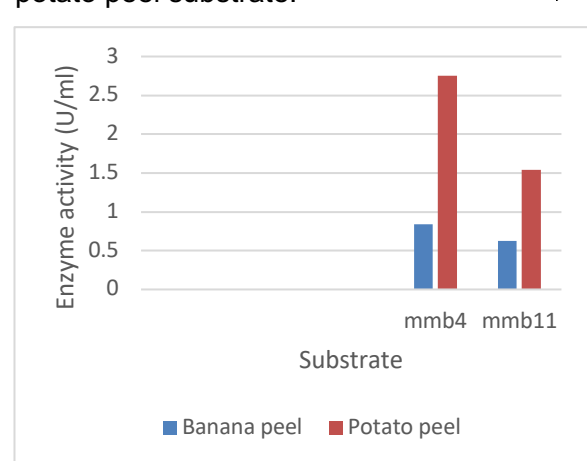
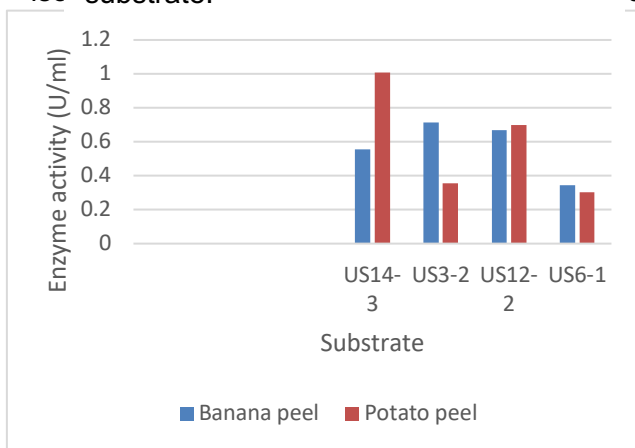
Sampling site	Substrate	Sample code	Species name	Enzyme activity (U/ml)
Ussangoda coast	Banana peel	US14-3	<i>Bacillus subtilis</i>	0.56
		US3-2	<i>Bacillus subtilis</i>	0.71
		US12-2	<i>Bacillus stratosphericus</i>	0.67
		US6-1	<i>Bacillus atlitudinis</i>	0.34
	Potato peel	US14-3	<i>Bacillus subtilis</i>	1.00
		US3-2	<i>Bacillus subtilis</i>	0.36
		US12-2	<i>Bacillus stratosphericus</i>	0.69
		US6-1	<i>Bacillus atlitudinis</i>	0.30
Mahapelessa hot spring	Banana peel	mmb4	<i>Bacillus subtilis</i>	0.84
		mmb11	<i>Bacillus subtilis</i>	0.63
	Potato peel	mmb4	<i>Bacillus subtilis</i>	2.75
		mmb11	<i>Bacillus subtilis</i>	1.54



**Fig. 1:** Alpha-amylase activity of *Bacillus* spp. isolated from Ussangoda coast and Mahapelessa hot spring on banana peel substrate and potato peel substrate.

In this study, banana peels and potato peels were used as the substrates for the fermentation of bacteria. According to the results obtained from the Mahapelessa hot spring cultures, the alpha-amylase activity is higher on potato peel than on banana peel substrate (Figure 3). In Ussangoda coast cultures, the alpha-amylase activities of US14-3 (*Bacillus subtilis*) and US12-2 (*Bacillus stratosphericus*) samples were higher on potato peel than on banana peel substrate. But in US3-2 (*Bacillus subtilis*) and US6-1 (*Bacillus altitudinis*) samples, the alpha-amylase activity is higher on banana peel than on potato peel substrate (Figure 2). The sample mmb4 (*Bacillus subtilis*) isolated from Mahapelessa hot spring shows the highest alpha-amylase activity on both potato peel (2.75 U/ml) and banana peel (0.84 U/ml) substrates

(Figure 2). The sample mmb11 (*Bacillus subtilis*) isolated from Mahapelessa hot spring shows the highest alpha-amylase activity on both potato peel (1.5 U/ml) and banana peel (0.6 U/ml) substrates (Figure 3). The sample mmb4 (*Bacillus subtilis*) isolated from Mahapelessa hot spring shows the highest alpha-amylase activity on both potato peel (2.75 U/ml) and banana peel (0.84 U/ml) substrates (Figure 3).



potato peel substrate.

#### 4. Conclusions

According to the obtained results, Mahapelessa hot spring isolate mmb4 (*Bacillus subtilis*) shows a higher alpha-amylase activity at a relatively high temperature (44.5 °C) which is the optimum temperature of Mahapelessa hot spring. This study has also confirmed that potato peel is a good source of starch on which alpha-amylase-producing bacteria can act and therefore, can be used as the substrate for natural alpha-amylase production at a lower cost. The findings of this study show that industrially important alpha-amylases can be produced naturally with the use of natural habitats at a very low-cost using food waste. It also shows that the natural resources of Sri Lanka are of inestimable value.

#### References

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