Study of Hydrolase Activities from Germinated Ascosphaera Apis Spores

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Abstract. *Spores from Ascosphaera apis, a fungus causing chalkbrood disease of honey bees, were cultivated in Sabouraud Dextrose Broth (SDB) at room temperature. Spore germination and hydrolase activities in the liquid medium were detected every 3 hr. of cultivation. It was found that spore germination increased with the cultivation time and reached 32.78±0.51% at the 27th hr. The hydrolase activities increased at the initial stage of germination and then decreased. The maximum activities of amylase, lipase and protease at 6, 9 and 12 hr. of cultivation were 3.76±0.16 U/mL, 3.58±0.70 U/mL and 43.31±0.92 U/mL respectively.*

Keywords:

Ascosphaera apis, spore germination, amylase, lipase and protease activities.

1. Introduction

Ascosphaera apis (A. apis), a filamentous fungi, caused the chalkbrood disease in honey bee larvae. The dead bee larvae are mummified by fungal mycelium which give them a grayish or chalky color. Spores could be picked up by foraging bees at nectar, pollen, or water sources and passed on to larvae in their food. When the fungus spores ingested with the larval food, spores germinate in the lumen of the larval gut. The fungus then grows into hemocel and internal organs of the larvae which damages tissues, leading to the death of the larvae [1]. During developing and colonizing in the larvae's body, the fungus produces high proteolytic and lipolytic activity [2].

Previously, we have studied growth of A. apis in SDB and found hydrolytic enzyme activities, i.e. amylase, protease, lipase and chitinase, in the culture medium associated with the fungal growth [3]. In this study, relation between hydrolase (i.e. amylase, protease and lipase) activities and the fungal spore germination in SDB was determined.

2. Experimental

2.1 Material

This study used A. apis from Petchabun province (A. apis, Petchabun isolate). The fungus spore was kept in 0.85% sterile normal saline at 4-7ºC for 1 year.

2.2 Method

2.2.1 Determination of spore number

One milliliter of stock spore suspension was diluted with 5 mL of 0.85% steriled NaCl in a test tube which contained 10-15 pieces of glass beads (2 mm. diameter). The tube was shaken for 10 min to disperse the ascus to ascospore. 100 µL of the spore suspension was pipetted and put on a haematocytometer. The spore number was counted under a light microscope (400X).

2.2.2 Spore germination

An aliquot (0.5% mL) of stock spore suspension was inoculated in 80 mL of SDB pH 8.0 in a 250 mL. Erlenmeyer flask and incubated at room temperature (30-32 ºC). An aliquot (3 mL) of the culture was taken from the flask every three hours for hydrolase activity assays and to find % spore germination.

2.2.3 Hydrolase assays

Determination of all hydrolase activities was done in three independent experiments. In each experiment the assays were done in triplicated.

2.2.3.1 Amylase assay

Two aliquot (0.5 mL each) of 2 % starch were pipetted into two test tubes (one was sample tube, the other was substrate blank tube). Instead of 2% starch, 0.5 mL of SDB was pipette into the third tube (enzyme blank tube). All

three tubes were warmed at 60 ºC in a water bath for 5 min. For the sample tube, 0.5 mL of sample was added while 0.5 mL of distilled water was added in the enzyme blank tube and the substrate blank tube. The tubes were incubated at 60 ºC for 20 min. and then 3 mL of 3, 5- dinitrosalicylate (DNS) reagent was added in each tube. The tubes were kept in a boiling bath for 15 min. and then cooled with cold water before the absorbance of the solutions were measured at 550 nm. One unit of amylase was the amount of enzyme that hydrolyse 1 % soluble starch and gave reducing sugar equal to 1 µmole glucose within 1 min. under the assay condition.

2.2.3.2 Protease assay

Five millitres of 2 % casein was pipette in a test tube and warmed in a water bath at 37ºC for 10 min. One millitre of sample was then added and incubated further 10 min. The enzyme reaction was stopped by adding 5 mL of 10 % trichloroacetic acid (TCA) and kept at room temperature for 30 min. The precipitate was then filtrate by using Whattman no. 1filter paper. Two millilitres of filtrate was taken and mixed with 5 mL 0.4 % sodium carbonate and 1 mL of Folin-Ciocalteau reagent. The absorbance was measured at 660 nm.

One unit of protease was defined as the amount of enzyme that hydrolysed 1 % casein and gave 1 µmole tyrosine within 1 min. under the assay condition.

2.2.3.3 Lipase assay

Two hundred microlitres of sample solution was mixed with 800 µL 50 mM sodium phosphate pH 7 and 960 µL EtOH. Then, 40 µL of 23 mM (in 99% EtOH) p-nitrophenyl palmitate was added, mixed and kept at room temperature for 10 min. The reaction was stopped by adding 2 mL of 50 mM sodium carbonate, mixed and left for 10 min. The absorbance was measured at 410 nm.

One unit of lipase was the amount of enzyme that hydrolyse 1 µmole of p-nitrophenyl palmitate within 1 min. under the assay condition.

3. Results

3.1 Change of spore during germination

When the spore suspension in 0.85 % sterile NaCl was determined under light microscope, the ascospore was oval shape (Fig. 1A). After the spores were cultivated in SDB for 3 hr., the spore become round shape (Fig. 1B) and there appeared a tip of germ tube at the 9th hr. (Fig. 1C). The germ tube was longer whereas the spore shape still exist at the 18th hr. and the 27th hr. (Fig. 1D and 1E respectively). The germ tubes became to be mycelia which can be seen in the culture flask at the 33th hr. (Fig. 1F).

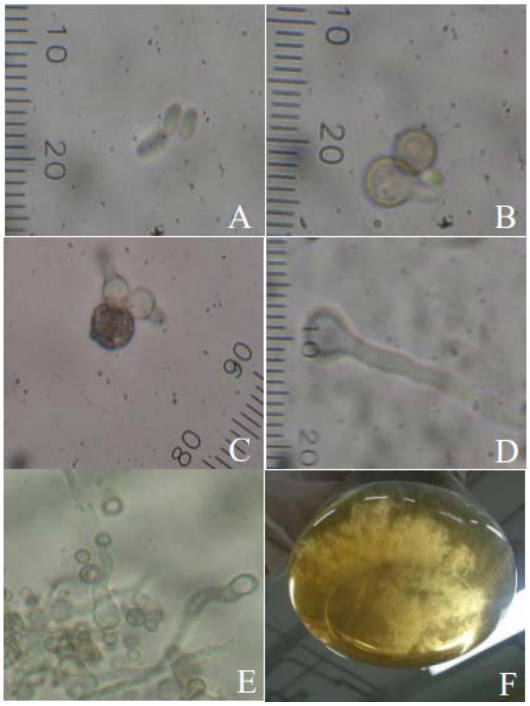

Fig. 1 Development of A. apis spores during germinated in SDB, 1A: spores before cultivation, 1B-1E: spores at the 3rd, 9th, 18th and 27th hr. of cultivation, 1F: fungal mycelium at 36th hr. of cultivation.

3.2 % spore germination

Spore germination was determined by counting germinated spores compared with one hundred spores which observed under light microscope (400X). Germinated spores were spores contain germ tube. The determination was done in three independent experiments.

From Fig. 2, there was a lag peroid before spore germinated since the % germination at the 3rd hr. of cultivation was less than 1%, then the germination was increase with cultivation time and reached 42.11±2.46% at the 36th hr. However, the fungal mycelium occurred at the 30th hr. and interfered the spore counting, therefore the counting ceased at the 36th hr.

3.3 Hydrolase activities

Even very few spores germinated in the first three hours of cultivation, hydrolase activities were already detected.

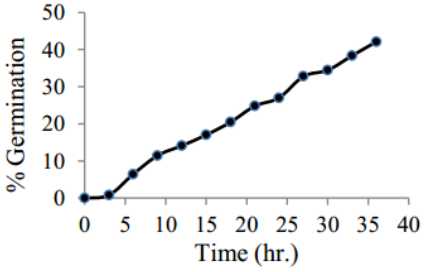

Fig. 2 % germination of A. apis spores in SDB.

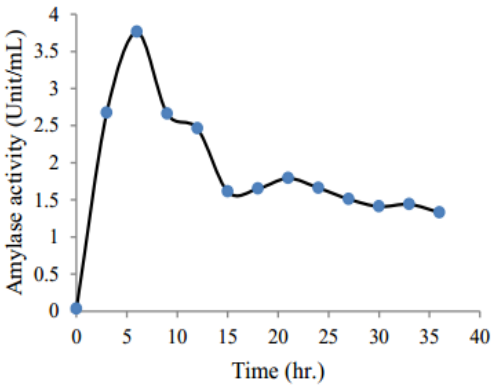

Fig. 3: Amylase activity in SDB from germinating A. apis spores.

From Fig. 3, amylase activity increased rapidly at the first three hours of cultivation and reached the maximum activity (3.76±0.16 unit/mL) at the 6th hr. and then declined until the 15th hr. and kept stable at around 1.50 unit/mL.

For protease activity, Fig. 4 the activity slightly increased to 8.03±0.09 unit/mL at the 3rd hr. and then decreased to 1.66±1.11unit/mL of the next 3 hr. of cultivation. At the 12th hr., the protease activity suddenly increased nearly 26 times to 43.3±0.92unit/mL and dropped to initial level (approximately 2 unit/mL) 3 hours later. The activity then gradually increased to approximately 10 unit/mL, and kept stable at the rest of cultivation time.

For lipase activity, Fig. 5, the activity increased gradually to reach the maximum (3.58±0.17unit/mL) at the 9th hr. and then decreased for the next 6 hr. of cultivation. The activity increased again at the 21th hr. then gradually decreased to basal activity (0.5 unit/mL) at the 36th hr.

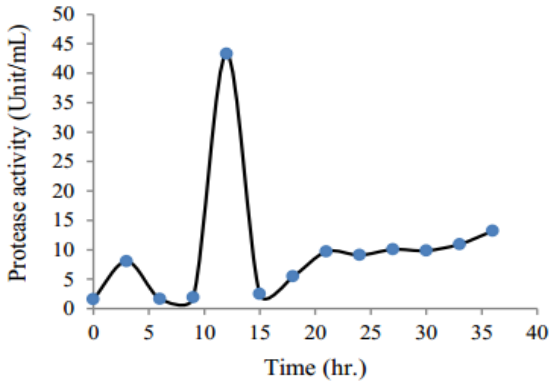

Fig. 4 Protease activity in SDB from germinating A. apis spores.

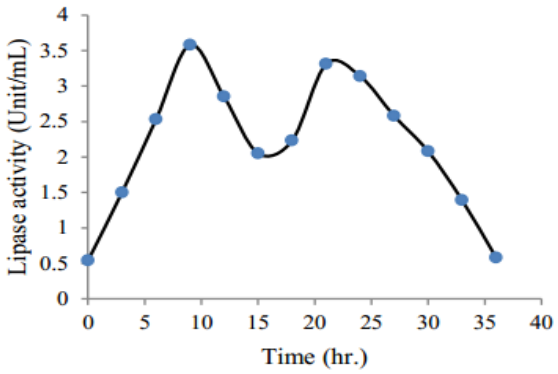

Fig. 5 Lipase activity in SDB from germinating A. apis spores.

4. Conclusion

When A. apis spores were transferred into SDB, It took 3 hr. before the spore began to germinated. The fungal mycelia were observed under light microscope at the 33th hr. and appeared in the medium culture at the 36th hr. The hydrolase activities were detected before spore germination and reached the maximum at the early stage of germination.

5. Acknowledgement

This research work was supported by the Department of Biotechnology, Faculty of Engineering and Industrial Technology, Silpakorn University, Nakhon Pathom, Thailand.

6. References

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Bibliography

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