Effect of agitation speed on morphological changes in *Aspergillus niger* hyphae in submerged fermentation system

Pengaruh kecepatan pengadukan pada perubahan morfologi "hyphae" *Aspergillus niger* dalam sistem fermentasi terendam

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Abstract

The correlation between the agitation speed and the morphological changes occurred in the fungal hyphae during the production of tannase in a submerged fermentation system has been studied. Under optimal agitation speed, loose fungal pellet formation favored enzyme production. Based on the SEM and TEM studies, it was found that there were significant correlations between the speed of agitation and the hyphal morphology including its internal structures, and the activity of enzyme production. This could be due to the excessive cell permeability related to abrasion by shear forces and also by oxygen limitation.

Key words: Agitation speed, fungal hyphae, tannase, submerged, fermentation system.

Abstrak

Telah dilakukan penelitian tentang hubungan antara kecepatan pengadukan dan perubahan morfologi dalam hipe fungi selama produksi tannase dalam sistem fermentasi terendam. Dibawah kecepatan pengadukan optimal kehilangan pembentukan "pellet fungal" diinginkan dalam produksi enzim.

Berdasarkan pada studi SEM dan TEM, ada korelasi nyata antara kecepatan agitasi dan morfologi "hyphae" termasuk struktur internal, aktivitas produksi enzim. Ini dikarenakan permeabilitas sel yang tinggi sehingga menyebabkan kerusakan oleh kekuatan pengguntingan dan terbatasnya oksigen.

Kata kunci : Kecepatan pengadukan, "hyphae fungal", tannase, sistem fermentasi terendam

Introduction

Tannin acyl hydrolase (E.C. 3.1.1.20) or also known as tannase is an inducible and a hydrolytic enzyme that hydrolyses ester and depside bonds in tannins such as tannic acid to produce gallic acid and glucose. Tannins are a group of water soluble phenolic compounds with different molecular mass, which form hydrogen bonds in solutions that result in the formation of tannin-protein complexes (Sabu *et al.,* 2005). Tannins are present in nutritionally important forage trees, shrubs legumes cereals and grains. Tannin can impair the digestive process by complexing with secreted enzymes and endogenous proteins. Tannase is produced mainly by fungi but also by yeast and bacteria.

Tannase is utilized in a number of industrial applications, such as in food, feed, beverage, brewing, pharmaceutical and chemical industries. The major commercial application of tannase are in the manufacture of instant tea and the production of gallic acid. Gallic acid is a key intermediate required for the synthesis of the antibacterial drug trimethoprim, used in the pharmaceutical industry (Sabu *et al.,* 2005). Tannase is also used as a clarifying agent in wine, fruit juices and coffee flavored soft drinks (Agbo and Spradlin, 1995). However, the main limitation in the development of the applications of xylanases is because of its low production level by the fungus. Furthermore, the cost of enzyme is one of the main factors determining the economics of a process. Therefore, method for improving the production of this enzyme in submerged cultures is inevitable to enable bulk production and indirectly enhances it application in industries. Several different strategies in order to enhance enzyme production by various *micro-organisms* have been successfully described (Marwick *et al.,* 1999) and these included the optimization through nutritional supplementation, addition of inducers and variation of cultural conditions.

Agitation speed is a very important factor in the fermentation process since it will increase the amount of dissolved oxygen in the cultivation medium. Erman reported that a maximum lignin peroxidase activity and a maximum fungal growth were achieved when the optimal agitation speed of 150 rpm was used (Erman, 1997). Excessive agitation would produce greater mechanical forces or hydrodynamic shear stresses and this condition is known to damage fungal mycelia and pellets (Porcel *et al.,* 2005) that lead to cell destruction, thus lowering the enzyme production. (Papagianni *et al.,* 2001) also found that the enzyme production was strongly affected by the agitation. At the higher agitation rates the enzyme production dropped. Agitation speed of the culture broth has a variety of effects on *micro-organisms*, including rupture of the cell wall, change in the morphology of filamentous *micro-organisms*, variation in the efficiency and rate of growth, and also variation in the rate of formation of the desired product (Porcel *et al.,* 2005).

The available literature on the effects of agitation speed on fungal mycelial morphology is mostly focused on mycelial cultures consisting of clumps and freely dispersed hyphae. At macroscopic level, one can distinguish between the filamentous growth form, where the hyphae are freely dispersed in the medium, and the pellet form, where mycelium develops spherical aggregates consisting of highly entangled networks of mycelium. On the other hand, some intermediate forms can be recognized such as flocculent or granular growth, while the term "clump" has been used either for the description of small loose mycelial aggregates present in a dispersed growth form or for big and heavy masses of aggregated mycelium (Bai *et al.,* 2003).

In general, little is known about the influence of mechanical forces or shear stresses on fungal hyphae (mycelia). Therefore, this work is aimed at studying the effect of agitation speed and also the correlation or relationship between the hyphal (mycelial) morphology, as influenced by agitation speed in the tannase production.

Methodology

Micro-organisms and development of inoculum

Aspergillus niger was maintained on potato dextrose agar slants supplemented with 1.0% tannic acid at room temperature (30 $\pm$ 2 °C). Fully sporulated slants were stored at 4°C in a refrigerator and subcultured once every three weeks. The

inoculum was prepared by adding 5.0 mL of sterile distilled water to agar slant, shake vigorously and counted in a Neubauer chamber. The spore suspension containing an average of 1.8 x 10^5 spores per mL was used as the inoculum.

Cultivation medium

Modified Czapek-Dox medium containing (%; w/v): 2.0% tannic acid, 0.25% NH_4NO_3, 0.10% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ and 0.05% KCl was used. Tannic acid was used as a sole carbon source. The cultivation process was carried out in 250 mL Erlenmeyer flasks with 100 mL of growth medium and inoculated with 1.3% (v/v) of 1.8x10^5 inoculum with initial pH medium of 6.0. The cultures were agitated at 0 rpm (static), 100, 130 and 200 rpm at 30°C for 6 days. All experiments were performed in triplicate.

Extraction and analysis of crude enzyme

The culture broth was filtered through GF/A glass fiber filters (Whatman) and Millipore membrane filters (Milipore) of pore size of 0.22 μm. Enzyme activity was expressed in units (U). One unit of tannase activity was defined as the amount of enzyme required to release 1.0 μmol of gallic acid per minute under standard reaction conditions.

Growth determination

Growth was measured based on the dry weight of the mycelia after filtration, followed by drying at 80°C for 24 hours until constant weight.

Scanning electron (SEM) and transmission electron (TEM) microscopy

The mycelia of the six days old *A. niger* cultures were collected from various agitation speed (0, 100, 150 and 200 rpm), washed several times with sterile distilled water and blotted dry in sterile filter papers (Whatman). The samples for SEM and TEM were prepared as described previously by Zacchi *et al(* 2000).

Results And Discussion

Industrial bioprocesses with filamentous fungi embrace the production of a majority of commercially important products of biotechnology, in the sense of quality as well as the diversity of metabolites. These are mainly the submerged culture processes, where a dynamic relationship existed between environmental conditions and the growth pattern of these modular *micro-organisms*. The morphology of filamentous fungi in submerged culture has been shown to play a critical role in many industrial fermentation and in commercial production of some metabolites. During submerged growth, many filamentous fungi may grow either as free mycelia or as pellets and the growth form is determined by a number of factors such as growth medium, size of inoculum and physical environment (Cho *et al.,* 2002). Therefore, distinct cultivation conditions result in different morphological and physico-chemical characteristics of fungal hyphal elements, and thereby their tendency to aggregate.

Table I shows the effects of agitation speed (0 rpm to 200 rpm) on tannase and growth production by *Aspergillus niger* in submerged fermentation system using shake flasks. The results revealed that the optimal agitation speed was 130 rpm which produced a maximal tannase activity of about 2.75 U/mL. However, the best fungal growth was achieved when the culture agitated at 100 rpm. The results suggested that the enzyme production was not growth dependent. The agitation speed that was higher than 130 rpm resulted in low enzyme production and this condition could be due to the fungal cell disturbances caused by shear stress or shear forces. On the other hand, lower agitation speed of less than 130 rpm resulted in low growth, which thus resulted in low tannase production. This could be due to low amount of dissolved oxygen in the cultivation medium.

Table I Effects of agitation speed on tannase production and growth of *Aspergillus niger*

Agitation speed (rpm)	Tannase activity (U/mL)	Growth (g/L)
0	1.75	2.15
100	1.98	4.35
130	2.67	4.10
150	2.54	3.55
200	1. 45	2.50

Table II: Effects of agitation speed on the morphology of *Aspergillus niger*

Agitation speed (rpm)	Fungal morphology	Diameter of pellet (mm)
0	Slimy and filamentous	None
100	Big rounded pellets	>10.0
130		4.0-6.0
150	Medium rounded pellets	2.0-2.5
200	Pelleted and filamentous Small pellet without Filamentous	<1.5

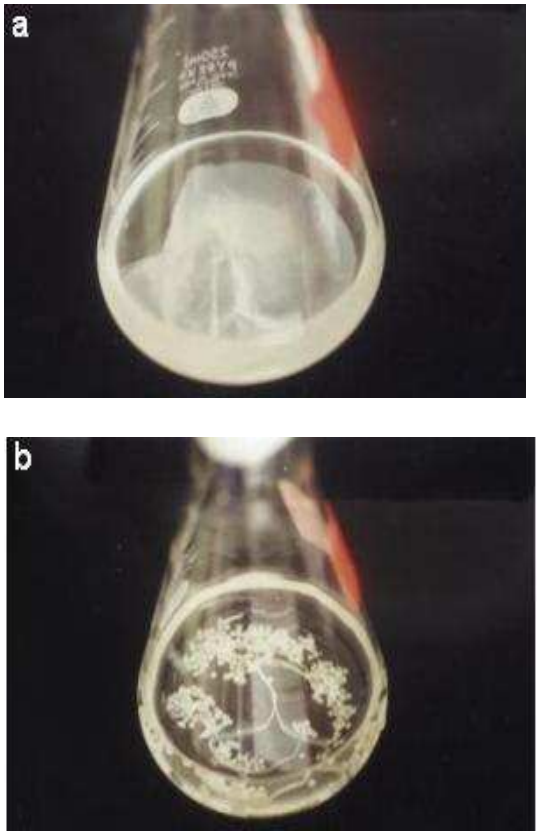

Figure 1. The morphological changes of *Aspergillus niger*. (A) Static (0 rpm) and (B) 150 rpm

Further studies on the effects of agitation speed from 0 rpm (static) to 200 rpm on tannase production showed that increasing agitation speed caused the fungal pellets to decrease in size but to increase in number per unit volume (Table II). At 0 rpm or static condition, the fungal mycelium developed a layer of fungal mat (Figure 1A), and as the agitation speed increased the fungal pellet formed (Figure 1B). The agitation speed also determined the size of fungal pellet formed and it seems that at 130 rpm, the medium size, rounded pellet produced maximal tannase production. The increase in agitation speed was expected to result in higher shear stress, causing the fungal to grow in pellets of smaller size and dense. The vigorous agitation speed suppressed both of the enzymes production and the caused was due to mechanical inactivation of the enzymes. Vigorous agitation seems to affect the catalytic activity of the enzymes. However, the addition of detergents such as Tween 20, Tween 40, Tween 60, Tween 80, and 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS) were able to protect both purified lignin peroxidase and extent the enzyme in culture fluids (free of biomass) against mechanical inactivation that due to agitation. Addition of Tween 80 at the end of primary growth to agitated shake flasks containing either pelleted or immobilized mycelial cultures resulted in production and maintenance of high levels of lignin peroxidase activity over several days under conditions of high agitation. The similar condition should be happened here if the detergent was added.

The investigation was also carried out further using SEM and TEM , in order to study the morphological and internal structural alterations of the fungal hyphae during the agitation speed. Figure 2 shows SEM micrographs taken at various agitation speed after 6 days of cultivation. It was found that at 0 rpm (static) the fungal hyphae were cylindrical and loose in shape (Figure 2A). As the agitation speed increased the hyphae became thinner and denser (Figure 2B), with less fruiting bodies (Figure 2C). At 200 rpm (Figure 2D) only tight packing mycelia seen, with the presence of many minute spikes on

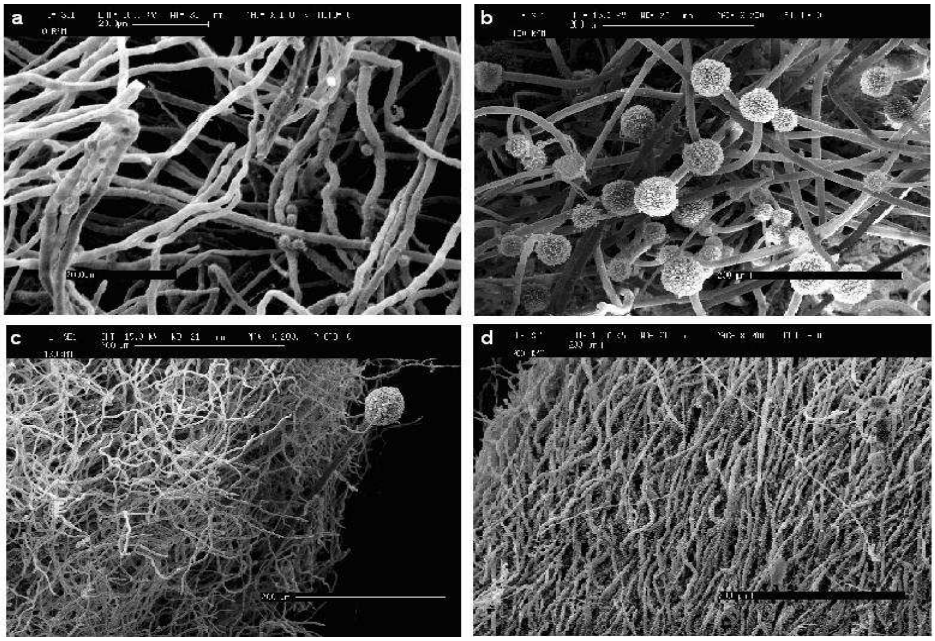

Figure 2. SEM micrographs of the effects of various agitation Speed on the mycelia of *A. niger*. (A) 0 rpm, (B) 100 rpm, (C) 130 rpm and (D) 200 rpm

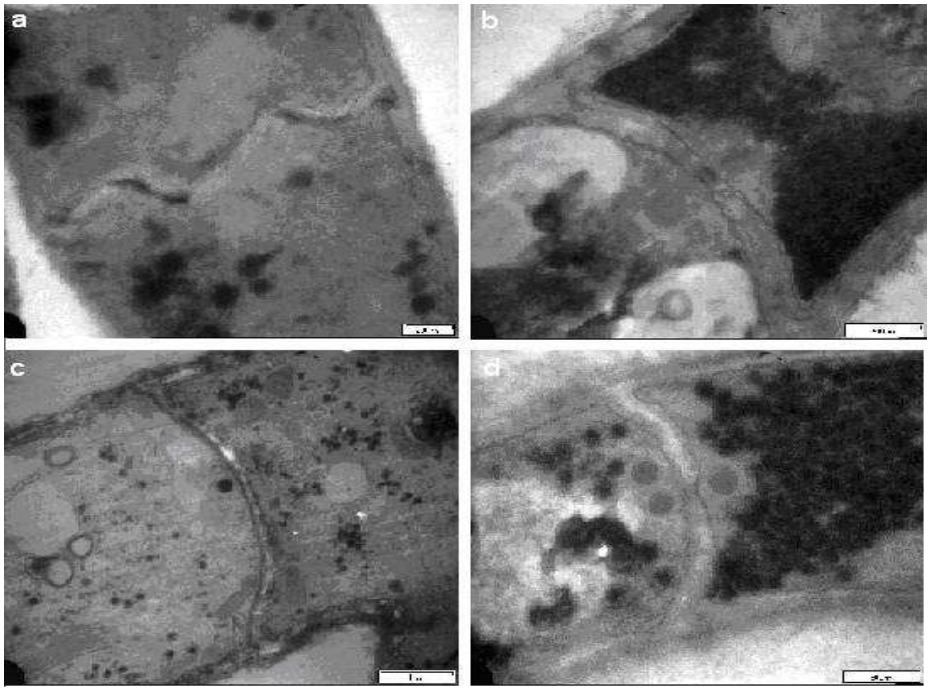

Figure 3. TEM micrographs of the effects of various agitation speed on the mycelia of *A. niger*. (A) 0 rpm, (B) 100 rpm, (C) 130 rpm and (D]) 200 rpm

them. In order to study the alterations occurred in the fungal hyphal cells, the TEM was carried out. Figure 3 shows the TEM micrographs of the hyphae at different agitation speed. At 0 rpm, the normal hyphal conditions seen (Figure 3A). At 100 rpm (Figure 3B), many vacuoles and mitochondria seen. At 130 rpm (Figure 3C), there were more mitochondria presence and this related to the higher enzyme production. However, at 200 rpm (Figure 3D), there were many small vesicles presence and lack of mitochondria.

The results of this study revealed that even though the agitation speed over 130 rpm could provide higher amount of dissolved oxygen in the culture broth and might enhance the enzyme production, but due to higher shear stress and forces, as well as higher abrasion, the enzyme production dropped. (Cho *et al.,* 2002) also found similar conditions with their fungus, *Kluyveromyces marxianus* during the red pigment production in a batch bioreactor (Cho *et al.,* 2002). Santisteban and Filho found that agitation, aeration and shear stress as key factors in their innulinase production (Santisteban and Filho, 2005). They concluded that higher agitation and aeration caused higher shear stress and those conditions could lead to cell death rates, that eventually affecting the innulinase production. However, the addition of detergents such as Tween 20, Tween 40, Tween 60, Tween 80, and 3-[(3-cholamido-propyl)dimethylammonio]-1-propanesulfonate (CHAPS) into the growth medium perhaps could be able to protect the enzymes against mechanical inactivation that due to agitation.

Conclusion

As a conclusion, there was of course correlations between agitation speeds and enzyme production in *Aspergillus niger*. The results obtained from this study revealed that interaction between dissolved oxygen and mechanical stress seems to define the tannase production, and should be considered in the cases of process optimization and scaling up. Mechanical stress gave greater impact on the cells and could alter the cell internal structures, thus lower the enzyme activity.

Acknowledgements

The Ministry of Healty of Indonesia is thanked for financial support to H.S. H.S. also thanks the Ministerio de Educacion Cultura, Spain for a contract as Foreign Young Research in Spain. We thank the Spanish Ministry of Science and Technology (PM99-0125) and the Xunta de Galicia for critical reading of the manuscript.

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