Appl Biochem Biotechnol (2011) 165:1682–1690
DOI 10.1007/s12010-011-9387-8

Influence of Agitation Speed on Tannase Production and Morphology of *Aspergillus niger* FETL FT3 in Submerged Fermentation

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Received: 1 June 2011 / Accepted: 12 September 2011 /
Published online: 27 September 2011

Abstract Agitation speed was found to influence the tannase production and fungal growth of *Aspergillus niger* FETL FT3. The optimal agitation speed was at 200 rpm which produced 1.41 U/ml tannase and 3.75 g/l of fungal growth. Lower or higher agitation speeds than 200 rpm produced lower enzyme production and fungal growth. Based on the SEM and TEM micrograph observation, there was a significant correlation between agitation speed and the morphology of the fungal mycelia. The results revealed an increase of the enzyme production with the change of the fungal growth morphology from filamentous to pelleted growth forms. However, the exposure to higher shear stress with an increasing agitation speed of the shaker also resulted in lower biomass yields as well as enzyme production.

Keywords Agitation speed · Tannase · *Aspergillus niger* FETL FT3 · Fungal morphology

Introduction

Tannin acyl hydrolase (E.C.3.11.20) or commonly known as tannase is an inducible enzyme that catalyzes the breakdown of ester and depside bonds present in hydrolysable tannin or gallic acid esters liberating glucose and gallic acid [1]. It cleaves the ester linkage between galloyl groups present in various compounds such as epigallocatechin and epigalloyl to catechin gallate [2]. The major commercial applications of tannase are in the manufacturing of instant tea, coffee-flavored soft drinks, fruit juices, beer, and wine where it is used to clarify the water-insoluble precipitates [3, 4]. Furthermore, it is also used to produce gallic acid and propyl gallate, where gallic acid is used in the pharmaceutical industry for the synthesis of antibacterial drugs. Even though the application of tannase is

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very wide for mankind, the main limitation in the development of tannase is due to its low production level by the microorganisms especially fungi. Furthermore, the cost of its production is one of the main factors determining the economics of a process. Therefore, procedures for improving the enzyme production in submerged cultures are inevitable to enable bulk production and finally enhance its application in industries. Several different methods have been developed in the attempt to enhance tannase production by various microorganisms, and these included the improvement through nutritional supplementation, addition of inducers, and variation in physical parameters [5, 6].

The effect of agitation speed on enzyme production is an important factor affecting the successful progress of submerged fermentation in a flask system, which can provide adequate mixing, mass, and heat transfer as well as improve dissolved oxygen. However, agitation speed also creates shear forces that can give a variety of effects on the microbial cell especially fungal cells [7]. These include the morphological changes to the fungal culture used especially in damaging the cell structure, variation in fungal growth, and product formation [8]. The purpose of this study was therefore to evaluate the influence of agitation speed on tannase production as well as morphology of *Aspergillus niger* FETL FT3 in submerged fermentation using a flask system.

Materials and Methods

Fungal Culture and Inoculum Preparation

A. niger FETL FT3 which was isolated from the dumping sites of tannin-rich barks of *Rhizophora apiculata* at the mangrove area in Perak, Malaysia, was used throughout this study. The fungal culture was maintained on 2% (*w*/*v*) malt extract agar slant supplemented with 0.01% (*w*/*v*) tannic acid at 30 °C for 5 days aerobically until sporulated, before storing it at 4 °C until further used. The subculturing was performed every 3 weeks to maintain its viability.

The inoculum was prepared by adding 5.0 ml of sterile distilled water to the agar slants and shaking vigorously. The spore suspension obtained was adjusted to 1×10^7 spores/ml using a hemocytometer slide chamber (Neubauer, Germany) and used as the inoculums.

Cultivation Medium

The fermentation was carried out in a 250-ml Erlenmeyer flask containing 50 ml of Czapek-Dox liquid medium [9] containing (percent; *w*/*v*): 0.25% $NaNO_3$, 0.1% KH_2PO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.05% KCl, and 1.0% tannic acid (as a sole carbon source). The pH of the medium was adjusted to 6.0, and 1.0% (1×10^7 spores/ml, *v*/*v*) of the inoculum was added in to it. The cultures were agitated at speeds of 0 (static), 50, 100, 150, 200, and 250 rpm using a rotary shaker (Certomart-H, B. Braun, Germany) at 30 °C for 6 days. All the experiments were performed in triplicates.

Enzyme Extraction

The cultivation medium was filtered through GF/A glass fiber filter (Whatman, UK) to separate supernatant from fungal mycelial mass. The cell-free culture filtrate was then filtered again using a membrane filter with a pore size of 0.22 μm (Millipore, USA) and was regarded as a source of tannase.

Tannase Activity Determination

Extracellular tannase activity was determined according to the method described previously by Deschamps et al. [10]. Enzyme activity was expressed in units per milliliter, and 1 U of tannase was defined as the amount of enzyme required to release 1.0 μmol of gallic acid per minute per milliliter of fermentation broth under standard assay conditions.

Growth Determination

Growth was determined based on the dry weight of the fungal mycelia after filtration using GF/A glass fiber filter, washing three times with distilled water, and finally drying at 65 °C until constant weight.

Scanning Electron and Transmission Electron Microscopy

The 6-day-old fungal mycelia were harvested from various agitation speeds of the rotary shaker (0, 50, 100, 150, 200, and 250 rpm), washed three times with sterile distilled water, and then blotted dry in Whatman filter papers. The samples for SEM and TEM were prepared as described previously by Darah and Ibrahim [7] and Lim and Darah [11].

Results

In a submerged culture using a flask system, the agitation speed was found to be a critical factor influencing both the fungal growth and tannase production. As shown in Fig. 1, the enzyme production and fungal growth were increasing as the agitation speed increased (from 0 rpm up to 200 rpm); however, at the agitation speed beyond 200 rpm, there was a drastic drop in the enzyme production and fungal growth. The maximum tannase production of 1.41 U/ml and fungal growth of 3.75 g/l were recorded at 200 rpm. The agitation speed higher or lower than 200 rpm exhibited lower tannase activity as well as lower fungal growth production. The results suggested that the tannase production by *A. niger* FETL FT3 was growth dependent.

Table 1 shows the influence of the agitation speed on tannase activity, fungal growth, and fungal morphology of *A. niger* FETL FT3 in submerged fermentation using a flask

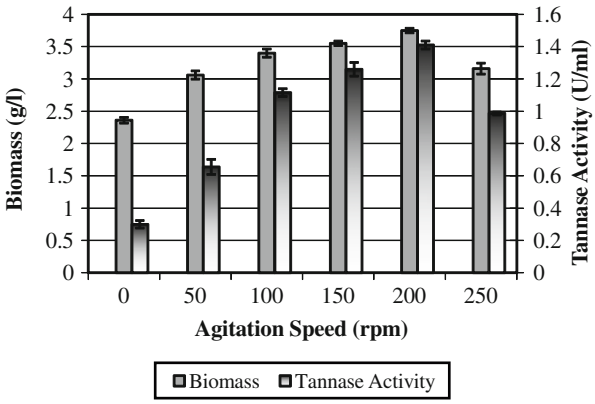

Fig. 1 Influence of agitation speed on fungal growth and tannase production by *A. niger* FETL FT3

Table 1 Influence of the agitation speed on tannase activity, fungal growth, and fungal morphology

Agitation speed (rpm)	Fungal morphology	Diameter of pellet	Tannase activity (U/ml)	Growth (g/l)
0	Slimy and filamentous	None	0.30	2.36
50	One big pellet with filaments	5.6 cm	0.66	3.06
100	One big pellet	4.9 cm	1.12	3.40
150	One small pellet	3.4 cm	1.26	3.55
200	Pelleted and filamentous	3.0–6.0 mm	1.41	3.75
250	Mixture of spherical and elongated pellets	≤4.0 mm	0.99	3.16

system after 6 days of cultivation. At 0 rpm (static condition), a well-growing mycelial mat was observed on the surface of the cultivation medium; however, with the agitation speed increased, the mycelial mat turned slowly to become circular pellets. The findings from this study indicated that liquid surface fermentation (with mycelial mat) was not suitable for tannase production as the enzyme production was the lowest. The cells were observed to form circular pellets during the agitated period irrespective of the agitation speed. The agitation speeds of 50, 100, and 150 rpm produced single pelleted growths that were big (5.6 cm), medium (4.9 cm), and small (3.4 cm), respectively. The optimized agitation speed which produced the highest tannase production and fungal growth was at 200 rpm, where the spherical pelleted growth between 3.0 and 6.0 mm formed. The agitation speed at 250 rpm produced smaller spherical and elongated fungal pellets at ≤4.0 mm. Similarly, these morphological changes of the *A. niger* FETL FT3 culture at different agitation speeds are shown in Fig. 2. Figure 2a shows the fungal mycelial mat formed on the cultivation medium when no agitation (static) was applied. Figure 2b, c, and d shows the circular fungal pellets formed when 50, 100, and 150 rpm agitation speeds were used, respectively. Figure 2e shows when the culture was agitated at 200 rpm, showing spherical pelleted fungal growth of 4.0–6.0 mm, while Fig. 2f shows the mixture of spherical fungal pelleted (≤4.0 mm) and elongated growth forms when agitated at 250 rpm.

In order to have a closer look at the influence of agitation speed on fungal morphology, the SEM study on the fungal morphology was carried out. Figure 3 shows the SEM micrograph of the fungal mycelia at different agitation speeds. Figure 3a shows the morphology at 0 rpm, where cylindrical and highly branched mycelia with some fruiting bodies were formed. As the agitation speed increased, the fungal mycelia became denser and formed many branches as shown in Fig. 3b (50 rpm), c (100 rpm), and d (150 rpm). At 200 rpm, the mycelia were highly branched and possessed numerous small projections (Fig. 3e) which actually were the new branches that formed as a result of the agitation speed. However, at the agitation speed higher than the optimum speed, the mycelia became denser and thinner (Fig. 3f) with minute projections.

Figure 4 shows the TEM micrograph of the cross sections of the hyphae at different agitation speeds. At static and low speeds up to 150 rpm, a large portion of the hyphae was observed to be occupied by numerous vacuoles with the cytoplasm closely pressed against the cell wall (Fig. 4a, b, c, and d, respectively). As the agitation speed increased to 200 rpm, unorganized masses of cytoplasmic granules (electron-dense granules) were observed widely distributed throughout the entire cytoplasm (Fig. 4e). However, at the agitation speed of 250 rpm, the vacuoles became fragmented, and unorganized masses of cytoplasmic granules were located near the cell wall (Fig. 4f).

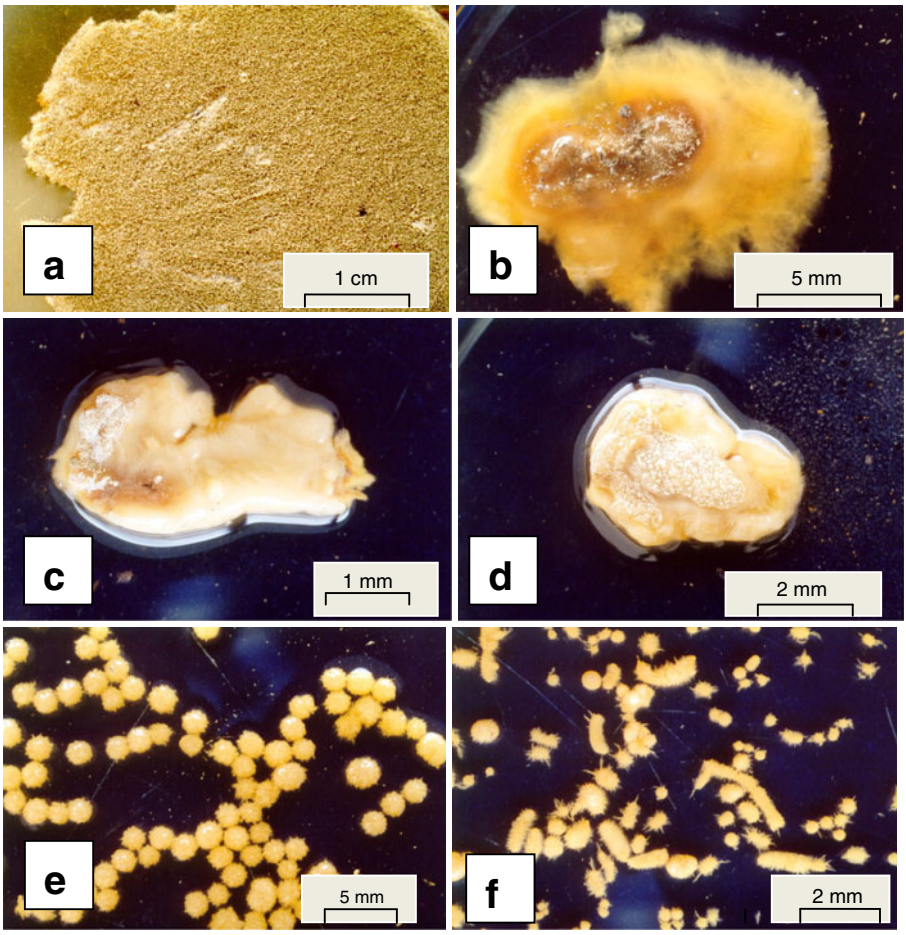

Fig. 2 Mycelial morphology of *A. niger* FETL F3 at different agitation speeds after 6 days of cultivation. **a** Static condition, **b** 50 rpm, **c** 100 rpm, **d** 150 rpm, **e** 200 rpm, and **f** 250 rpm

Discussion

The morphology of filamentous fungi in submerged fermentation is shown to have a critical role in metabolite production especially if the fermentation system involved agitation. During submerged fermentation, filamentous fungi may grow either as free fungal mycelia or as pellets, and their growth form is determined by physical and chemical factors [12]. The results obtained from this study proved that agitation speed plays an important role in influencing tannase production as well as fungal growth. The growth of filamentous fungi depends both on the condition of physical and chemical parameters. The agitation speed below 200 rpm resulted in low fungal growth and low tannase production. This condition could be due to the low amount of dissolved oxygen in the cultivation medium. As the agitation speed increased from 0 rpm (static) up to 200 rpm, it caused both the tannase production and the fungal growth to increase. However, at the agitation speed above 200 rpm, the tannase activity and the fungal biomass also dropped. This condition showed that agitation can also damage mycelial pellets just as it damages particular hyphae. Since

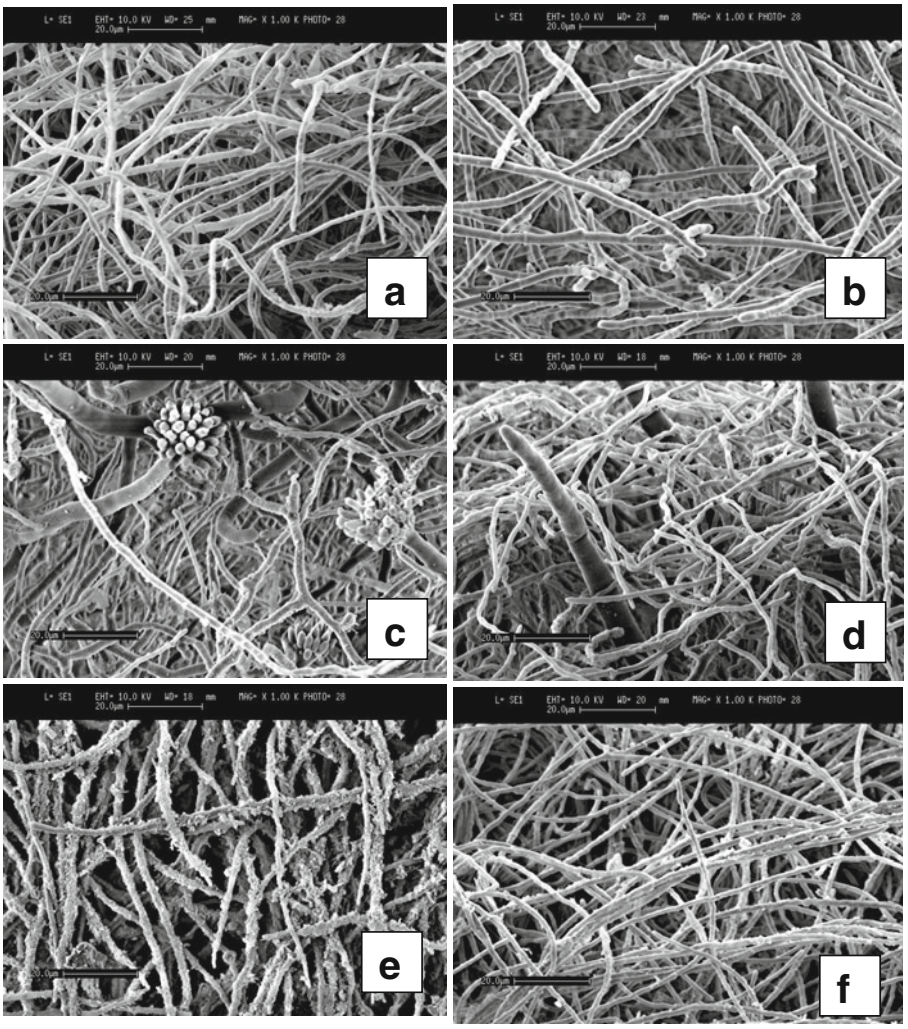

Fig. 3 TEM micrograph of the mycelial morphology of *A. niger* FETL F3 at different agitation speeds. **a** 0 rpm (static condition), **b** 50 rpm, **c** 100 rpm, **d** 150 rpm, **e** 200 rpm, and **f** 250 rpm

hyphae grow only at the tip, the total biomass concentration in submerged cultures increases by the number of branches and by the formation of new fragments, caused by shear stress. Seth and Chan [13] also explained that at lower agitation rates, the inadequate mixing of the broth towards the later stages of growth affected the enzyme synthesis by accumulation of gallic acid on the surface of the mycelium, while the drastic fall in enzyme activity at higher agitation rates was largely due to shearing effect on the mycelium. In the same way, Barthomeuf et al. [14] and Pourrat et al. [15, 16] have found that excessive aeration favored the oxidation of tannins and thus had inhibiting effects on the biosynthesis of the tannase. Furthermore, changes in the morphology of microorganisms caused by different agitation speeds were also found to influence tannase production and growth of the organism [17, 18].

In the present study, the cells were observed to form rounded to spherical pellets during the entire culture period irrespective of agitation condition. The smaller and denser pellets

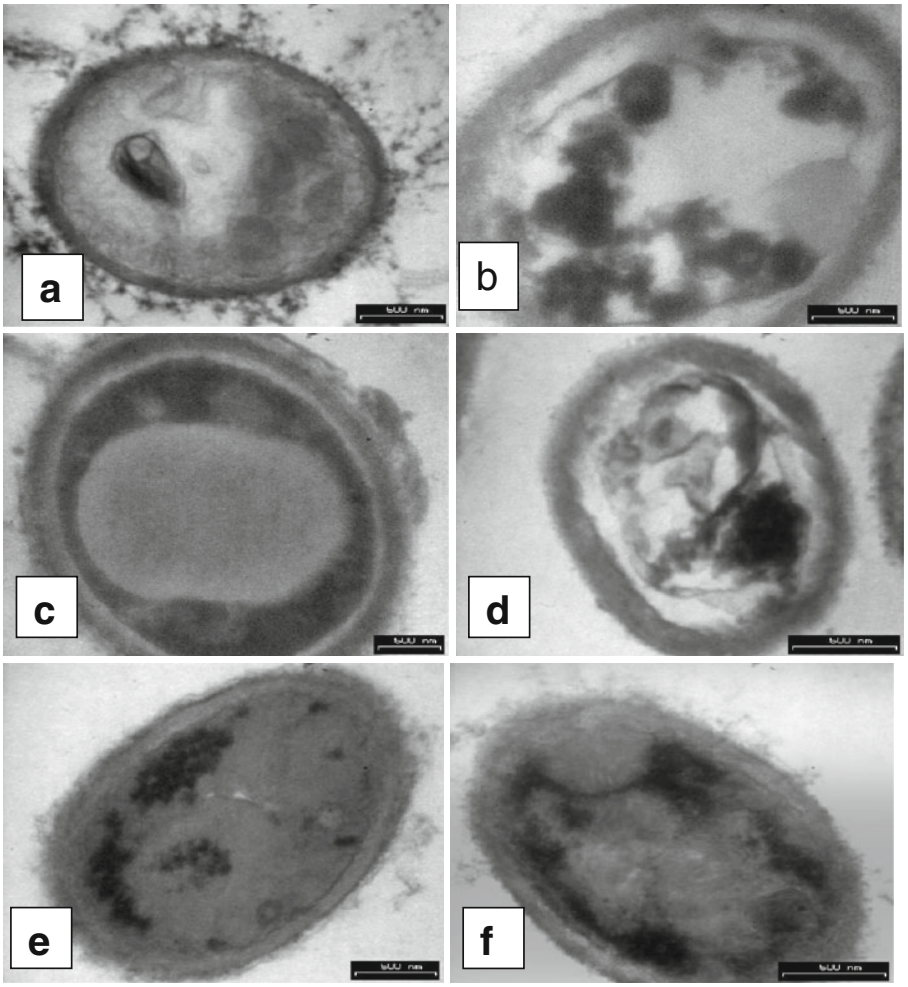

Fig. 4 TEM micrograph on the cross sections of *A. niger* FETL FT3 mycelium at different agitation speeds. **a** Static condition, **b** 50 rpm, **c** 100 rpm, **d** 150 rpm, **e** 200 rpm, **f** 250 rpm

of high agitation speed (200 rpm) were found to cause increased production of mycelial biomass and tannase as compared to larger pellets formed under low agitation speed. This is possibly because the central regions of larger pellets undergo central autolysis as a result of substrate and oxygen limitation [18] and therefore causes the pellet core to become inactive (dead). Therefore, viable mycelia of large pellets existed only on the surface of the pellet, and a high fraction of the pellet was inactive for tannase production. On the other hand, smaller pellets with high hyphal density observed at high agitation speeds of 200 rpm and above possibly had higher frequency of active mycelia and therefore enhanced tannase production. This indicates that small pellets as opposed to large ones are more effective in the production of tannase by *A. niger* [18]. However, at too high agitation speeds above 200 rpm, biomass and tannase production decreased. This indicated that too high a speed could have created shear forces which lead to the destruction of the mycelium and consequently to cell damage [18, 19]. The increase in agitation speed than the optimal ones

was expected to produce higher shear stress that causes the fungi to grow in pellets of smaller size that are denser. Similar findings were reported by Darah and Ibrahim [7, 20] where *Phanerochaete chrysosporium*, a white-rot basidiomycetous fungus, produced extracellular lignin-degrading enzymes as part of its idioplasmic system. They also found that vigorous agitation speed suppressed the enzyme production, and it was due to mechanical inactivation of the enzymes. To overcome this problem, they suggested the addition of Tween 80 at the end of primary growth in an agitated shake flask containing pelleted fungal cultures. The addition of Tween 80 was able to protect the enzyme against mechanical inactivation that was due to agitation [21].

Vacuoles are generally reported to contain hydrolytic enzymes or as a site for accumulation of various metabolite substances [22]; however, to date, no studies on the hyphal vacuole content of tannase-producing microorganisms have been reported. As agitation speed increased to 200 and 250 rpm, unorganized masses of cytoplasmic granules (electron-dense granules) were observed widely distributed throughout the entire cytoplasm. Brieger [23] indicated these kinds of granules are ribosomes consisting of proteins and ribonucleic acid. Ribosomes are small particles of about 17 to 23 nm in diameter which may occur in clusters or aggregates called polysomes or polyribosomes [22], are sites at which amino acids are linked together to form proteins, and have been reported to be abundant in the cytoplasm of metabolically active cells [24]. Thus, this study clearly shows that agitation causes morphological changes in cells during fermentation and in turn influenced tannase production.

Conclusion

The results obtained from this study revealed the influence of agitation speed on tannase production, fungal growth, and morphology of *A. niger* FETL FT3 in submerged culture, confirming the interaction between mechanical (shear) stress and dissolved oxygen. However, mechanical stress, or also known as shear stress, has a great impact on cells and may alter its internal structures, thus lowering the tannase production.

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