Biotechnology Letters **26:** 41–44, 2004.

Quantitative characterisation of the morphology of *Trichoderma harzianum* cultured in shake-flasks and containing Tween 40

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Received 22 July 2003; Revisions requested 19 August 2003; Revisions received 22 October 2003; Accepted 24 October 2003

Key words: fungi, morphology, shake-flasks, *Trichoderma*, Tween

Abstract

Image analysis was used to measure the effect of Tween 40 on the morphology of *Trichoderma harzianum*. The percentage of pellets was maximal (93%) with Tween at 0.2 ml l^{-1}, whereas the maximal proportion of dispersed mycelia (40%) was with Tween at 1.6 ml l^{-1}. The particle median diameter decreased from 2 to 0.5 mm as Tween concentration was increased and was inversely correlated to the biomass dry weight. Adding pre-determined aliquots of Tween 40 to the culture medium can be used to define the morphological characteristics of *Trichoderma harzianum* in submerged shake-flask culture.

Introduction

Tween compounds are polyesters of sorbitan and fatty acids from which, generally, only the fatty acid moiety can be used as a carbon source (Wynn & Ratledge 2000). They are widely used as surfactants in mycelial cultures. Filamentous fungi, growing in submerged cultivation, are able to develop complex morphologies, which have been classified into three major groups: pellets, mycelial aggregates (clumps) and freely dispersed mycelia (Thomas 1992). Parameters exerting a strong influence upon morphology, such as inoculum level (Johansen *et al.* 1998, Domingues *et al.* 2000), agitation rate (Jimenez-Tobon *et al.* 1997, Johansen *et al.* 1998) and culture medium composition (Domingues *et al.* 2000), have been used to establish certain morphological types during cultivation. This is important as mycelial morphology has been related to the overall performance of submerged cultures, mainly affecting metabolite productivity and rheology (Braun & Vecht-Lifshitz 1991, Metz & Kossen 1997). Tween 80 promotes dispersed growth and inhibits pellet formation in *Trichoderma reseei* cultures (Domingues *et al.* 2000). Previous studies however have not quantitatively characterised the morphology of the fungi studied nor have they assessed the effect of a wide range of surfactant concentrations. Furthermore, and with the exception of one paper (Galindo *et al.* 2003), no reports are available characterising the morphology of *Trichoderma* spp. during the later stages of cultivation in shake-flasks, which are widely used in biotechnology research. In this work we quantitatively analyse the influence of Tween 40 (as a culture supplement) on morphology and biomass synthesis of *Trichoderma harzianum* cultured in shake-flasks.

Materials and methods

Strain and culture conditions

Trichoderma harzianum IMI 206040 was used. Spore concentration in all experiments was 10^3 spores ml^{-1}. Mycelial growth was performed in 500 ml shake-flasks with 100 ml culture medium with the following composition (g l^{-1}): glucose (50), $(NH_4)_2SO_4$ (5), KH_2PO_4 (7), $NaHPO_4$ (2), $MgSO_4 \cdot H_2O$ (1.5), $CaCl_2 \cdot 6H_2O$ (0.067), $ZnSO_4 \cdot 7H_2O$ (0.0001), $FeCl_3 \cdot 6H_2O$ (0.008). Tween 40 (95% polyoxyethylenesorbitan monopalmitate). Prior to sterilization, the initial pH of the culture was adjusted to 5.6 with concentrated H_3PO_4 and was not controlled through cultivation. The culture flasks were capped with silicon closures and agitated at 29 °C at 200 rpm (shaking amplitude = 2.5 cm). The harvest time was at 108 h. All experiments were done at least in duplicates.

Biomass concentration

Biomass dry weight were obtained by filtering samples (5–10 ml) through a 0.45 μm pore size membrane, dried out and weighted.

Morphological measurements

Morphological features of mycelial particles and the percentage of dispersed mycelia, clumps and pellets in each sample were determined using the image analysis system Image-Pro Plus (v4.1, Media Cybernetics, USA). At least 300 randomly chosen mycelial particles were measured for each sample. Individual morphological parameters measured were mean diameter, roundness and compactness. Roundness describes the deviation of mycelial particles from a true circle, and compactness gives an estimation of hyphal entanglement density. Both parameters were calculated as described by Thomas (1992). As a general trend, we observed positively skewed size (mean diam., Figure 3) distributions as Tween 40 concentration increased, hence we decided that the median (and not average) particle diameter was a representative parameter of the most common mycelial particles at each experimental condition. Based on our visual criteria, we established the following values for morphological classification: particles having a mean diameter less than 0.3 mm were considered dispersed mycelia. Clumps were considered as particles bigger than 0.3 mm, and with compactness less than 0.99; pellets were defined as particles having a compactness value between 0.99–1 and roundness lower than 6.

Results and discussion

The percentage of each morphology obtained at the different Tween 40 concentrations are shown in Figure 1. There was a rapid transition from pelleted growth to more dispersed morphologies at Tween 40 concentrations higher than 0.4 ml l^{-1}. There seems to be a limit for clumped growth at around 60%, as higher Tween concentrations did not change the proportion of this morphology.

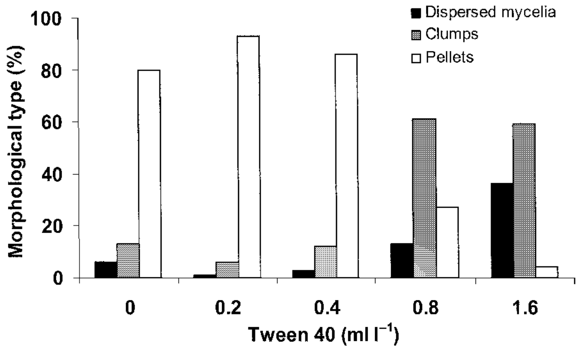

Fig. 1. Morphology composition obtained at each Tween 40 concentration.

In the control culture (without Tween), enlarged and star-like pellets were formed (Figure 2a). In the medium with 0.2 ml Tween l^{-1}, well-defined spherical pellets were formed, most of them with a compact and smooth structure possessing narrow filamentous growing ring (Figure 2b). These observations agree with the lowest mean roundness (high circularity) value obtained (Figure 4b). Compared with the control run, a narrower bell shaped distribution was obtained with 0.2 ml Tween l^{-1}, indicating greater pellet homogeneity in the medium (Figure 3).

Culture broths with 0.4 ml Tween l^{-1} exhibited a range of morphologies. In this case, one population consisted of loose pellets However, pellet sizes were slightly smaller than those obtained in both the control medium and that with 0.2 ml Tween l^{-1} added. Further increases in surfactant concentration resulted in the most pronounced effect on fungal morphology with filamentous growth being highly promoted (Figure 2d).

Biomass production increased in proportional to the Tween concentration (Figure 4a). Biomass production with 1.6 ml Tween l^{-1} was almost 1.5-fold higher than the control with the medium being practically utilised at its maximum theoretical biomass yield (10 g l^{-1}). A similar response of biomass yields increasing with a decreasing particle size for this *Trichoderma* strain was observed (using rich medium and higher inoculum concentration) when agitation rate was increased from 200 to 300 rpm (Galindo *et al.* 2003), indicating that oxygen transfer (which depends directly on volumetric power input) also plays a role as a morphology-controlling factor in this fungus.

The effect of surfactants on fungal morphology seems to depend on the strain and on properties of the surfactant. For example, Tween 80 increased aggrega-

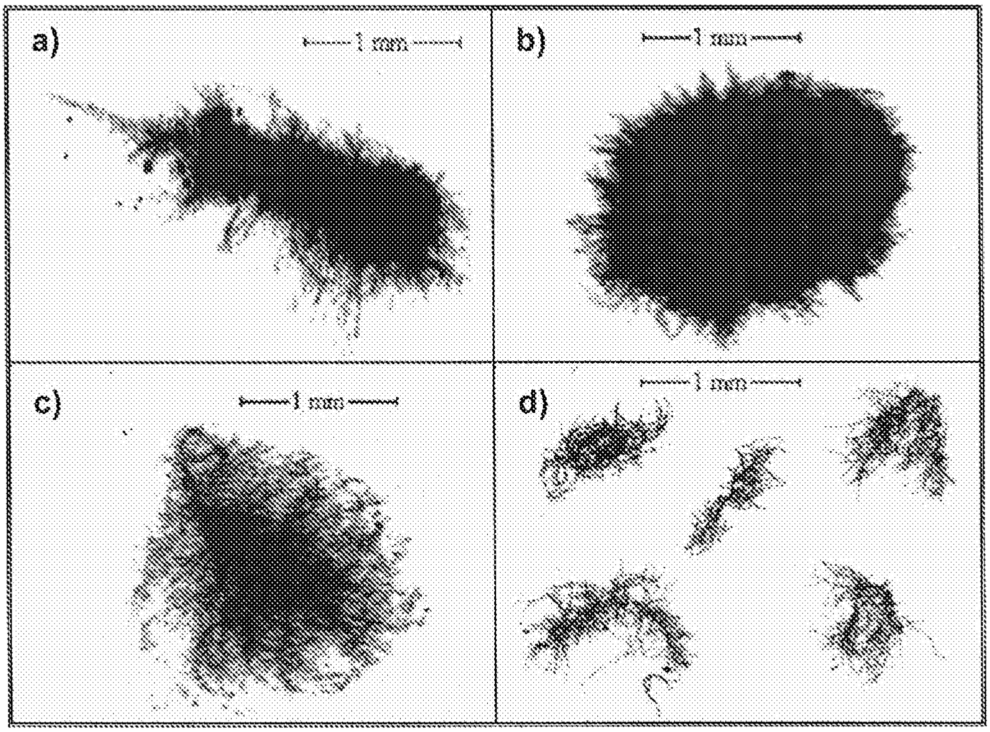

Fig. 2. Representative mycelial particles obtained at the different Tween 40 concentrations: (a) 0, (b) 0.2, (c) 0.4 and (d) 0.8 and 1.6 ml l^{-1}.

tion and formation of large pellets with a loose structure in *Aspergillus niger* (Metz & Kossen 1997). Conversely, Tween 80 promoted filamentous growth and inhibited pellet formation in cultures of *Trichoderma reesei.* Cellulase production also increased, presumably through an increased permeability of the cell membrane, allowing more rapid secretion of enzymes (Domingues *et al.* 2000). This promotion effect on metabolite excretion has also been observed for other fungi (Golberg & Stieglitz 1985). In this work, Tween 40 induced pellet formation at low concentrations and filamentous growth when higher than 0.8 ml l^{-1}. On the other hand, high Tween concentrations could favour the incorporation of Tween molecules into the cell wall, thus inhibiting spore aggregation, which is known to promote a more filamentous morphology.

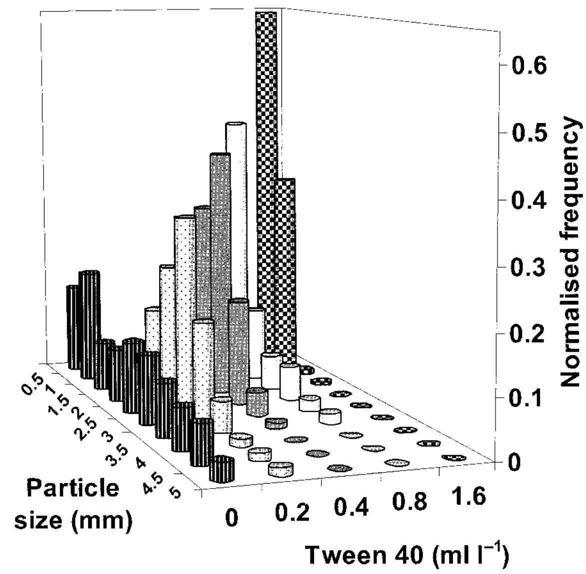

Fig. 3. Mycelial particle size distributions at each Tween 40 concentration.

Conclusions

Low spore inoculum of *Trichoderma* has been known to favour pellet formation in submerged fermentations. However, it was also possible to shift from pelleted to dispersed growth by manipulating the amount of Tween 40 added to the culture. One direct effect of this morphological change was a 50% increase in biomass yield. Production of a defined morphology could be useful for producing pre-inocula of a particular type (say for a bioreactor) as well as more fundamental

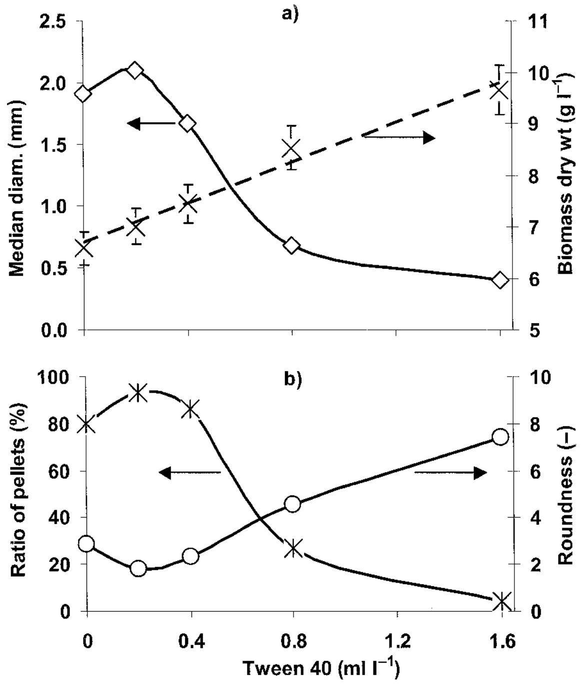

Fig. 4. Influence of Tween 40 supplementation in: (a) mycelial median particle size and biomass dry weight, and (b) pelleted morphology percentage and mean roundness.

studies on fungal physiology. We concluded that a reproducible and convenient manipulation of *Trichoderma harzianum* mycelial morphology in shake-flask cultures can be achieved by controlled supplementation with Tween 40.

Acknowledgements

The authors would like to thank the financial support of DGAPA IN 117202. The invaluable technical support of Gabriel Corkidi-Blanco, Leticia Vega-Alvarado and Blanca Taboada is acknowledged. Helpful discussions with José Manuel Baizabal are also acknowledged.

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