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Characterization of morphological forms of *Aspergillus carbonarius* and the effect of inoculum size on raw starch digesting amylase (RSDA) production

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The effect of spore inoculum level and mycelia morphology on raw starch digesting amylase production by *Aspergillus carbonarius* in submerged culture was investigated. Shake flask cultures were inoculated directly with spore concentration range of 10^4 to 10^9 spores/ml. Morphology was differentiated into, pellets, clumps, branched and free filaments. Results on the percentage morphologyical occurrences revealed that for all tested inoculum a gradual change in morphology was observed as innoculum level increased from 10^4 to 10^9 spores/ml though pellets predominated. A decrease in the mean main and total hyphal length, mean hyphal growth unit and marginal increase in mean number of tips/mycelium was observed in the mycelia trees as inoculum levels increased from 10^4 to 10^9 spores/ml. In the aggregated forms, there was a decrease in the mean area, perimeter, roughness and equivalent diameter while mean compactness increased as inoculum increased from 10^4 to 10^9 spores/ml. Irrespective of the predominating form a change in morphology was observed. RSDA production appeared to be related to spore inoculum level. Maximum yield was observed at fermentation inoculated with 10^4 and 10^5 spores/ml where pellets were most dominant. The impact of spore inoculum size on the resulting mycelia morphology was marginal but had a significant effect on RSDA production.

Key words: *Aspergillus carbonarius*, raw starch digesting amylase, fungal morphology and inoculum.

INTRODUCTION

When grown in submerged culture, fungi exhibit diverse morphological forms, ranging from dispersed mycelia filaments to densely interwoven mycelia masses referred to as pellet (Papagianni and Mattery, 2006; Papagianni, 2004) The particular form exhibited is determined not only by the genetic material of the fungal species but also the nature of the inoculum, medium constituents, as well as the culturing conditions (Kossen, 2000). There are cases, where control of mycelia morphology was considered a prerequisite for successful metabolite production. In some processes a particular morphological form may be preferred to achieve maximal production

(Znidarsic et al., 2000; Van Suidjam et al., 1980; Steel et al., 1954). Filamentous growth of *Aspergillus niger* for instance is preferred for pectic enzyme production, while the pelleted form gives optimal yield during citric acid production (Steel et al., 1954; Kristensen and Bulluck, 1988; Gomez et al., 1988). Inoculum quality (size, shape and type) and morphology affects filamentous fermentation. Van Suijdam et al. (1980) reported that *A. niger pellets* would only form at inoculum sizes below 10^8 spores/ml, while according to Calam (1987), *Penicillium chrysogenum* forms pellets at inoculums sizes below 10^4 spores/ml, but at higher inoculums levels, dispersed growth prevails (Braun and Vecht-Lifshitz, 1991). Studies on the two extreme morphological forms (pellets and free filaments) have shown that, a morphological form may account for the bulk of the biomass like the case of *P. chrysodenum* where clump morphology accounted for 90%

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of the biomass (Parker and Thomas, 1990; Tucker et al., 1992). Papagianni and Mattey (2006) observed a clear transition from pellets to dispersed morphological forms in citric acid fermentation by *A. niger* at inoculum concentration range of 10^4 to 10^9 spores/ml, with pellets constituting at least 95% of biomass at 10^4 spores/ml inoculum level, while clumps constituted 90% at inoculum levels at 10^6 and 10^7 spores/ml.

The application of image analysis since the 1990s has enabled the extraction of quantified information in the detailed characterisation of various morphological forms. Tucker and Thomas (1992) described quantitatively the transition from pelleted to disperse forms of growth of *P. chrysogenum* as inoculum level rose towards 10^5 spores/ml of medium. Papagianni and Mattey (2006); Pamboukian et al. (2002); Papagianni and Mooyoung (2001) have used the same method to study the characterisation of *A. niger* and *Streptomyces olindensis* in submerged fermentations. Different software's are now available for the characterisation of morphological forms, Nair et al. (2011) applied fluorescent indicators to analyse intracellular calcium and morphology in the filamentous fungi *P. chrysogenum*. The applicability of using Ca^{2+} sensitive dye to quantify $[Ca^{2+}]$ in *P. chrysogenum* cultures and concurrent application of dye-loaded hyphae for morphological analysis using the imaging software filament tracer (Bitplane) was investigated. Essential quantitative mycelia information including the length and diameter of the segments and the number of branch points were obtained using their application based on the three dimensional data. In the study of Ruhl and Kues (2009), an image analysis system for fungal pellets was developed using the commercial software system analysis and the protocol was evaluated in morphological studies of pellet formation in submerged cultures of basidiomycete *coprinopsis cinerea*.

Several important morphological studies have been carried out with filamentous fungi and streptomyces, in organic acid and enzyme production, but there has not been any work on raw starch digesting amylase fermentation and *Aspergillus carbonarius*. The aim of this investigation was to observe the trend in morphology as well as the effect of changing spore inoculum level on the resulting mycelia morphology of *A. carbonarius* in submerged cultures for raw starch digesting amylase production. Also we tried to characterise and quantify the mycelia morphology. The Moticam 1000 digital microscope camera (motic image plus software) was used for studies on morphology of the fungus.

MATERIALS AND METHODS

Microorganisms, inoculum and media preparations

A. carbonarius var (Brainer) Thom IMI 366159 (University of Nigeria, Nsukka) was used throughout this work. The organism was maintained on potato dextrose agar (PDA)

slants subcultured every 3 months. *A. carbonarius* maintained on PDA slants was transferred to fresh PDA plates and incubated at 30°C for 5 days. Spores wer e collected from mature culture plates and spore suspensions were diluted with sterile medium to make a range of concentrations in the order of 10^4 to 10^9 spores /ml of media.

The fermentation medium comprised (gL^{-1}): Raw cassava starch (*Manihot utilissima* Crantz), 20; yeast extract, 5; KH_2PO_4, 7; $CaCl_2.6H_2O$, 0.3; and $MgSO_4.7H_2O$, 0.3 in distilled water and autoclaved at 121°C for 15 min at 15 psi.

Culture conditions (shake flask cultures)

The microorganism produced aerial spores. Inoculum preparation was by means of washing 3 plates of dense growth with 10 ml of fermentation medium and transferring the spores and mycelium aseptically into 250 mL flask, the spore content was approximately 10^{11} spores /ml. Five hundred millilitre (500 mL) flask containing 100 ml fermentation medium was inoculated with spores in the concentration range of 10^4 to 10^9 spores/ml. Fermentation was carried out with rotary shaking at 100 rpm and 30°C for 144 h.

Amylase activity assay

Raw starch digesting amylase activity was assayed using reaction mixture containing 0.5 ml of 1% (w/v) of raw starch in 0.1 M citrate-phosphate buffer pH 6.0, 0.5 ml of crude enzyme solution and 0.1 ml toluene incubated at 40°C for 10 min. Reducing sugars released after incubation were estimated by DNS method of Miller (1959). One unit of the amylase activity was defined as the amount of enzyme that produced reducing sugar equivalent to 1 µmol of glucose from raw starch per min under the assay conditions.

Image analysis and processing

Image capture was carried out via a semiautomatic image analyser Moticam 1000 digital microscope camera UK, (Motic Images plus 2.0 ML) with a USB 2.0 cable. Mounted on a microscope (Optika, Italy) connected to a PC.

Samples for morphological characterization were taken at 4, 6, 8, 12, 18, 24 h and subsequently at 24 h interval for 144 h. Samples were immediately fixed with an equal volume of fixative as described by Parker and Thomas (1990), 13 ml of 40% w/v formaldehyde and 5 ml glacial acetic acid added to 200 ml of 50% w/v ethanol. The fixed samples were further diluted with fixative and dilution was adjusted to separate the mycelia clumps on the microscope slide. Images were captured at 640 to 512
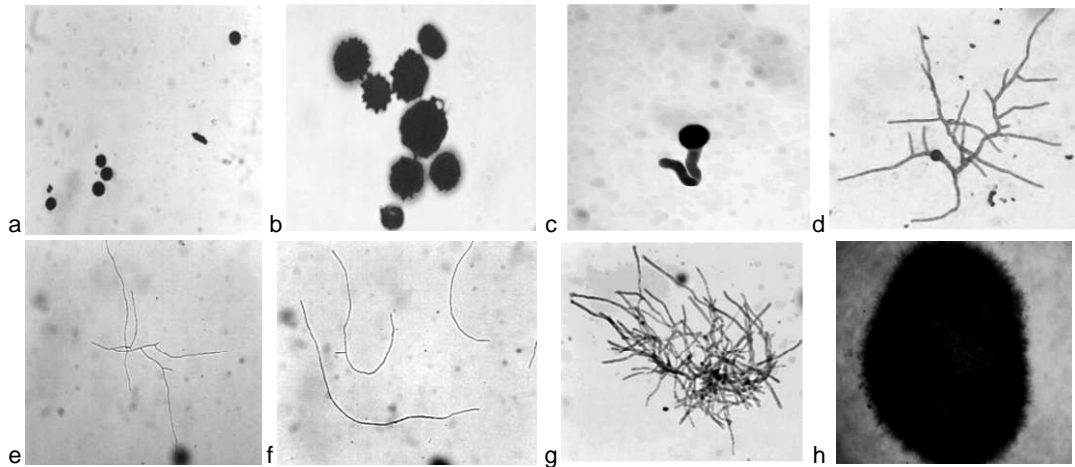
Figure 1. Different classes of morphology from submerged culture of *A. Carbonarius* fermentation: **a** spores of *A. carbonarius* at 0 h of fermentation; **b** swollen spores at 4 h; **c** germinating hyphae at 6 h; **d** branched hyphae at 12 h; **e f** freely dispersed mycelia at 24 h; **g**, **h** clump and pellet respectively at 24 h of fermentation.

pixals. A magnification of 40× and 100× was applied for measurements on mycelia particles.

Segmentation was performed to obtain a binary image; measurements were carried out on binary images and by adjustment of greyness levels. A filter was applied to the segmentation to show the ends and branching points and the total length was measured. Medium particles artefacts, debris and morphological particles which were touching the organism and could have biased the results were removed by filter and in some cases computer aided manual measurement was applied to measure and subtract false images.

Mycelial morphology was classified into unbranched, branched, entangled or clumped and pelleted. The morphological parameters measured were, mean main and total hyphal length, number of tips and hyphal growth unit (total length divided by number of growing tips) for the dispersed form. For the analysis of clumps/pellets the area, perimeter, compactness, roughness and diameter were measured. The roughness is given by the circularity factor using the equation (p2/4πA) and compactness was estimated by the ratio of the area of the hyphae in the clump/pellet to the total area enclosed by its actual outer perimeter (Tucker et al., 1992). Percentage morphology was determined by mycelia count.

RESULTS AND DISCUSSION

### The development of *Aspergillus carbonarius* morphology

Figure 1 shows different classes of morphology of *A. carbonarius* in submerged cultures. Results indicated that spores appeared swollen at 4 h of fermentation and a good number of them were agglomerated. It was observed that at 6 h period, germination had started with branched hyphae, while at 12 h branching was very apparent and permanent aggregates were detected. By inoculating directly with spores we were able to observe the period of spore swelling, germination and branching.

At 24 h, a morphological trend was established as they were in either pellets or free filamentous form depending on the spore inoculum level. Results obtained in this study are consistent with the observation of Papagianni and Mattey (2006), with respect to citric acid fermentation by *A. niger*.

As illustrated in Figures 2 to 7 varying the spore inoculum level resulted in different mycelia morphology at the end of growth phase in the batch culture. All levels of tested inocula were plotted against morphological parameters corresponding to dispersed or aggregated morphological form. For all the tested inocula pellets dominated but different morphological form were identified.

Figure 2 shows percentage morphology occurrence at 72 h for various inoculum levels. Data obtained revealed various morphological forms with pellets dominating in all the range of inocula evaluated. With 10^4 and 10^5 spore/ml inoculum level, pellets accounted for 95 and 90%. Inoculum levels of 10^6 and 10^7 spores/ml gave a mixture of pellets, clumps and dispersed mycelium with pellet dominating at 75 and 65%, respectively, clumps at 15 and 20% and dispersed at 10 and 15% respectively. Inoculation with 10^8 and 10^9 spores/ml revealed pellets to account for 50% of detected objects while the clumps and dispersed were 30 and 20% respectively. Tucker et al. (1992) in testing a method for fully automatic image analysis of mycelia morphology reported that over 90% of the batch culture consisted of clumped morphological forms. Papagianni and Mattey (2006) reported pellets to
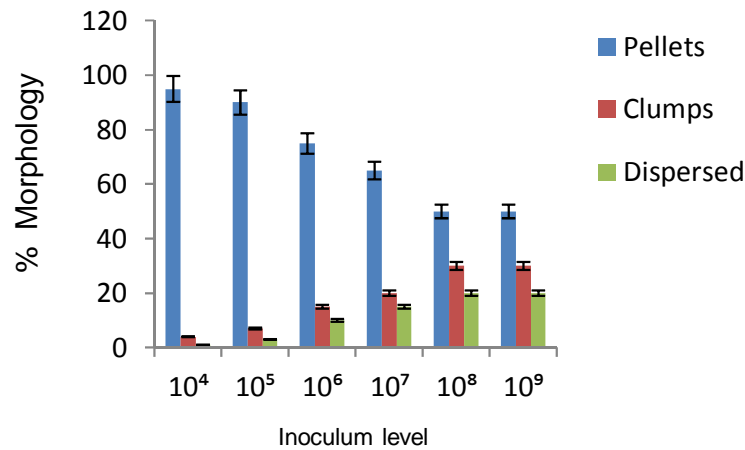
Figure 2. Percentage occurrence of different morphological forms of *A. carbonarius* in fermentation inoculated with spore inocula ranging from 10^4 to 10^9 spores/ml (72 h).
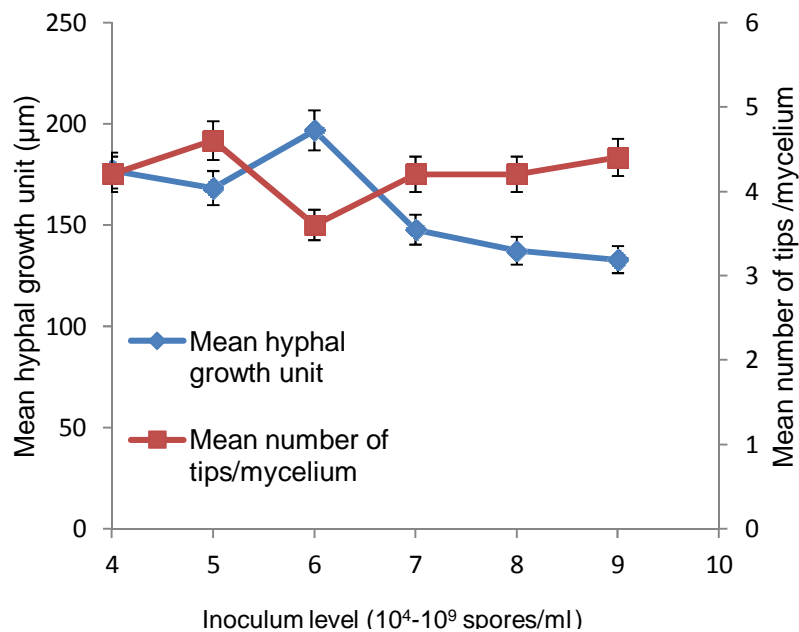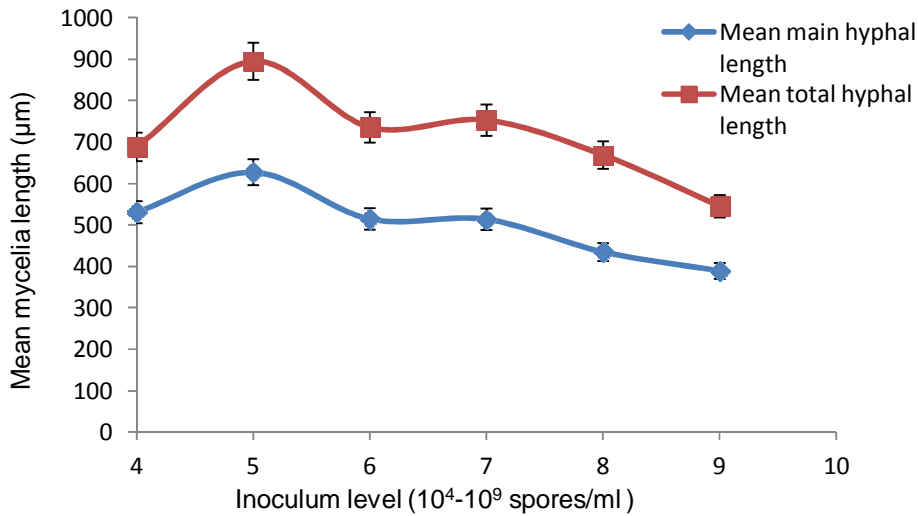

Figure 3. Effect of spore inoculum level on mean hyphal growth unit and mean number of tips at 72 h of fermentation with spore inocula ranging from 10^4 to 10^9 spores/ml.

account for 95 and 90% of mycelia growth with inoculum levels of 10^4 and 10^5 spores/ml, while clumps and dispersed morphology dominated in inoculum levels of 10^6 to 10^9 spores/ml. There was no sharp transition from pellet to dispersed in the present investigation, as reported by Papagianni and Mattey (2006) and Tucker et al. (1992) who studied the effect of inoculum level with spore inoculum range of 10^4 to 10^9 and 5×10^4 to 5×10^5 spores/ml, respectively, but rather the presence of all

morphological forms was observed in all the tested inoculum levels. However looking beyond the predominating form one can say that there was a gradual change from pelleted to disperse morphology. It is therefore possible that the agitation intensity influenced the rate of pellet formation in the case of the present study.

Figures 3 and 4 show the effect of spore inoculum levels on the dispersed morphological form. It can be
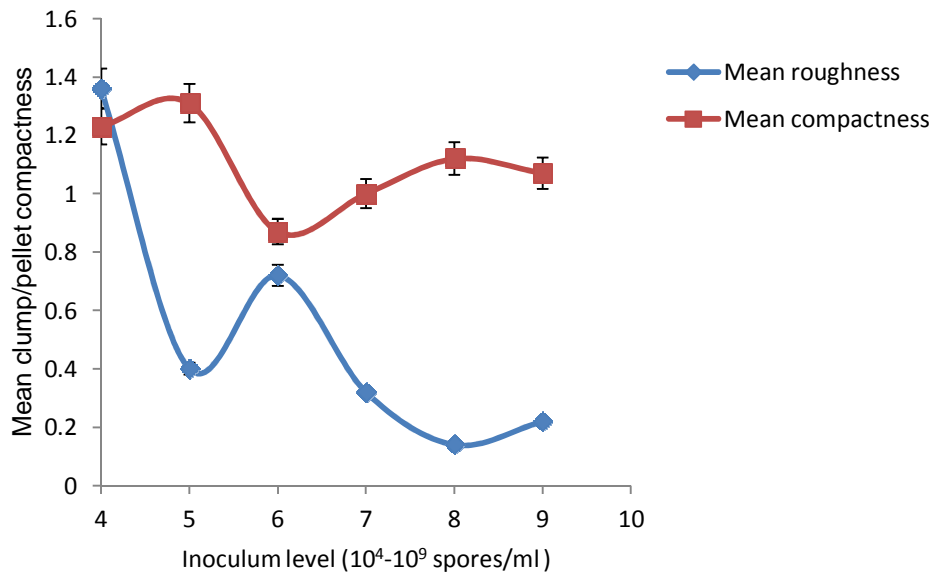
Figure 4. Effect of spore inoculum level on mean main hyphal length and mean total hyphal length of mycelium at 72 h of fermentation inoculated with spore's inocula ranging from 10^4 to 10^9 spore/ml
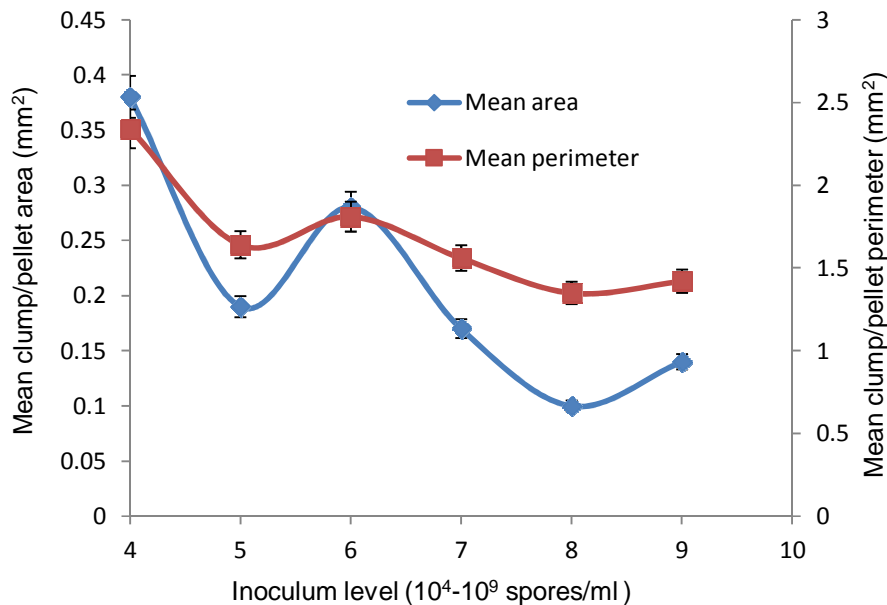

Figure 5. Effect of spore inoculum level on mycelia aggregation. Mean area and mean perimeter of clump/pellet at 72 h of fermentation inoculated with spore inocula ranging from 10^4 to 10^9 spores/ml.

deduced from the result that there was a decrease in the mean hyphal growth unit, and an increase in mean number of tips/mycelium as inoculum level increased from 10^4 to 10^9 spores/ml. There was a marked decrease in both the mean main hyphal length and mean total hyphal length of mycelium as inoculum level increased from 10^4 to 10^9 spores/ml. The results of this study showed that the impact of the spore inoculum level on the

detailed characteristics of a particular morphological form was marginal. Results of the mean number of tips/myceliium could be attributed to low agitation intensity (100 rpm), where there is little or no breakage of the mycelia particle. There are several reports which suggest that hyphae are shorter, thicker, and more highly branched at high agitation speed compared to low speed (Dion et al., 1954; Metz et al., 1981; Van Suidjam and Metz,

Figure 6. Effect of spore inoculum levels on mycelia aggregation. Mean compactness and mean roughness of clumps/pellets at 72 h of fermentation inoculated with spore inocula ranging from 10^4 to 10^9 spore/m.
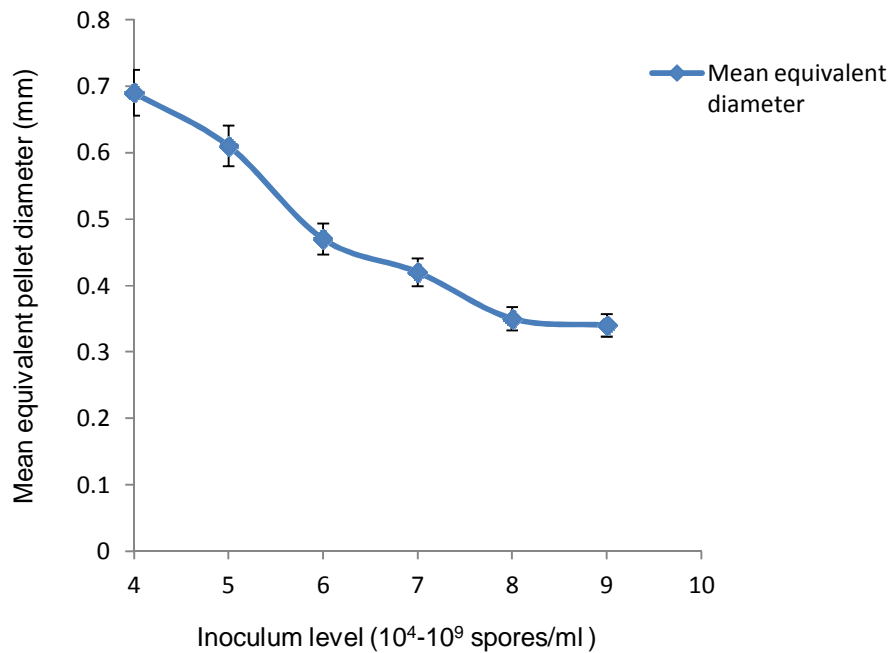
Figure 7. Mean equivalent diameter of pellets at 72 h of fermentation of spores inoculated with spore inocula ranging from 10^4 to 10^9 spores/m.

1981). Also Amanullah et al. (1998) found the mean number of tips to be higher at 550 rpm compared to 1000 rpm, which implied less highly branched hyphae at higher speeds. Papagianni and Moo Young (2002); Papagianni et al. (1999); Nielson et al. (1995), Belmar-Beiny and Thomas (1991); and Paul et al. (1994), reported that mycelia fragmentation common to fungal fermentation is associated with high agitation levels. Decrease in the mean hyphal growth unit; mean main hyphal length and total hyphal length as inoculum increased from 10^4 to
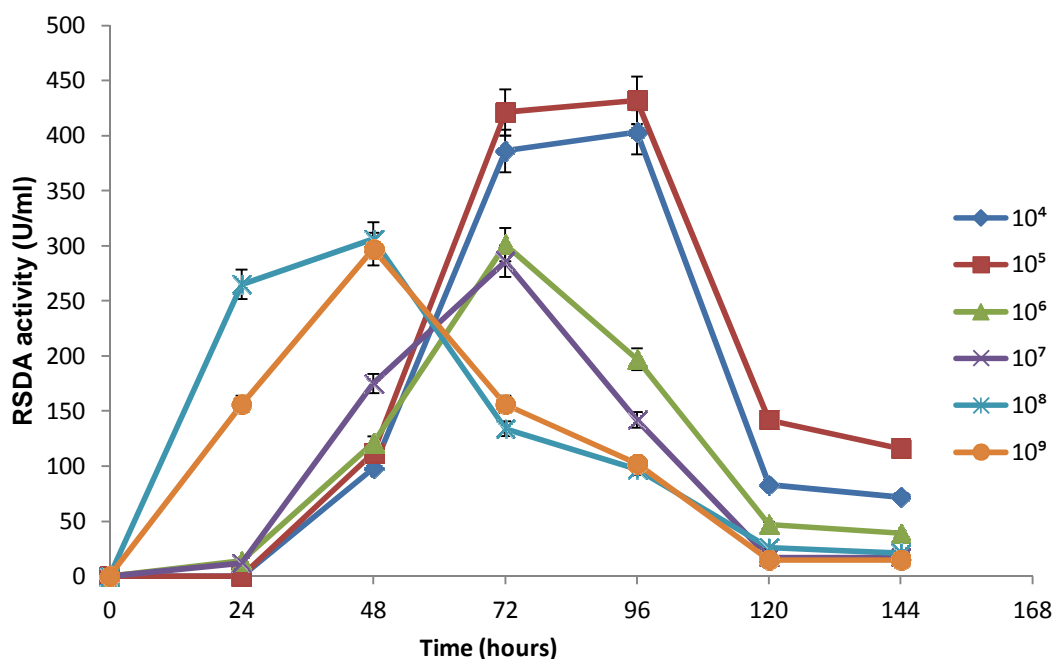
Figure 8. Time course of RSDA production by *A. carbonarius* with spore inocula ranging from 10^4 to 10^9 spores/ml.

10^9 spores/ml, may be attributed to the presence of mixed morphological objects (a mixture of pellets, clumps and dispersed morphology) resulting from higher inoculum levels. According to the results of Papagianni and Mattey (2006), Nielson et al., (1995), and Tucker and Thomas (1992) higher spore concentration yielded clumped and dispersed morphology.

Figures 5 and 6 show the effect of spore inoculum levels on mycelia aggregation; The results depict that there was a marked decrease in mean area and mean perimeter of clumps and pellets as inoculum levels increased from 10^4 to 10^9 spores/ml, while their roughness decreased and compactness increased as inoculum levels increased from 10^4 to 10^9 spores/ml. This is consistent with Tucker and Thomas (1992) for *P. chrysogenum* and Papagianni and Mattey (2006) for *A. niger*. They reported a large decrease in clump and pellet sizes and marked changes in compactness and roughness when inoculum levels were increased from 5x 10^4 to 5 × 10^5 spores/ml and 10^4 to 10^9 spores/ml, respectively.

Figure 7 shows mean equivalent diameter of pellets at 72 h of fermentation of spores inoculated with spore inocula ranging from 10^4 to 10^9 spores/ml. It revealed that mean equivalent diameter of pellets decreased as inoculum level increased from 10^4 to 10^9 spores/ml. Since pellets dominated in all levels of inoculums, the mean equivalent diameter (the diameter of a circle of the same area as the measured feature) of approximately 25 pellets was measured per sample and the variation reported as percentage error. Smaller sized pellets

resulted from the 10^8 to 10^9 spores/ml inoculum level, while larger pellets where observed in the lower inoculum of 10^4 to 10^5 spore/ml. The result of the decrease in pellet diameter differed with the report of Nielson et al. (1995) who found pellet diameter increasing with spore concentration in *P. chrysogenum*. They attributed the result to physical contact between hyphal elements at higher spore concentrations. However our report is in agreement with Papagianni and Mattey (2006), who observed that agglomeration leading to pellet formation, is determined by both the organism and the environmental conditions.

Figure 8 shows the results of RSDA production by *A. carbonarius* with spore inocula ranging from 10^4 to 10^9 spores/ml. The results revealed formation and release of RSDA to be related to spore inoculum level. At 24 h in higher inoculum level (10^8 and 10^9 spores/ml) RSDA was formed. The optimum yield of 306 and 297 U/ml was noticed at 48 h in fermentation inoculated with 10^8 and 10^9 spore/ml. At inoculum levels of 10^4 and 10^5, a gradual increase in yield was observed with an optimal yield of 403 and 432 U/ml at 96 h of fermentation and a sharp drop by the end of the fermentation. Inoculating with 10^6 and 10^7 spores/ml let to a gradual increase in RSDA with an optimum yield of 301 and 286 U/ml at 72 h of fermentation.

Conclusion

The use of Moticam camera for image analysis enabled

us to classify and to a reasonable extent quantify the effect of spore inoculum level on fungal morphology. We were able to identify all morphological forms in all tested inocula and besides the dominating morphological form and low agitation intensity there was a change from pellet to dispersed morphology as inoculum increased from 10^4 to 10^9 spores/ml. The impact of spore inoculum level on the characteristics of each particular morphological form produced by changing the concentration of spores was marginal. The present research indicated that adjusting spore inoculum level influenced mycelia morphology and RSDA production.

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