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Influence of Stirrer Speed on the Morphology of *Aspergillus carbonarius* var (Bainier) Thom IMI 366159 During Raw Starch Digesting Amylase Production

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ABSTRACT

Agitation speed had various influence on the performance of *Aspergillus carbonarius* during the production of raw starch digesting amylase. By means of image analysis the following parameters; mean hyphal growth unit, mean total hyphal length, mean number of tips/mycelium and mean convex perimeter (P) of clump/pellet were used to characterize the mycelia morphology. Amylase activity, protein content, biomass concentration and pH were determined. The study was aimed at investigating how agitation speed will affect mycelia morphology and culture parameters during raw starch digesting amylase production in shake flask cultures. Fungal morphology was greatly influenced by agitation, with large pellets predominating at 100 revolutions per minute (rpm) while clumped mycelia was predominant at 150 and 200 rpm. Increasing the agitation speed caused a reduction in mycelia particle sizes. Maximum Raw Starch Digesting Amylase (RSDA) activity was achieved at a shake speed of 100 rpm with minimum protein concentration. In contrast, maximum protein concentration and lower amylase enzyme activity was achieved at a shaker speed of 150 rpm. On the other hand, at the highest shaker speed of 200 rpm used in this study, highest biomass and intermediate amylase enzyme activity as well as intermediate protein concentration were observed. These results show that shaker speed will play important role in obtaining optimum results of the various different parameters (amylase activity, protein concentration and biomass production) during the production of amylase from *Aspergillus carbonarius*. In these experiments, shaker speed had little or no influence on pH of the media during RSDA production.

Key words: Morphology, *Aspergillus carbonarius*, amylase, agitation

INTRODUCTION

Amylases are very important and widely used enzymes, accounting for about 30% of world enzyme production (Van Der Maarel *et al*., 2002). They play very prominent role in biotechnology with wide range application which span over the textile industries, paper and cellulose, leather, detergents, beverages, conversion of starch to sugars, animal chow, fermentation, medical and pharmaceutical industries (Okolo *et al*., 2000; Pandey *et al*., 1999; Butzen and Haefele, 2008). Amylases are enzymes that breakdown starch to simple sugars (Akpan *et al*., 1999; Okolo *et al*., 2000) and they can be found in plants, fungi, yeast, bacteria and actinomycetes (Okoli *et al*., 2010; Jaiswal *et al*., 2011; Nwagu and Okolo 2011; Roohi *et al*., 2011; Joshi, 2011; Pandey *et al*., 2000),

however, the bacterial and fungal source of the enzyme are mostly applied in the industry (Pandey *et al*., 1999). The efficient production of amylases depends on several physical and chemical factors such as pH of reaction mixtures, temperature, medium composition, moisture, agitation, incubation period as well as nature of inoculum (Smith *et al*., 1990, Teodoro and Martins, 2000; Saxena *et al*., 2007; EL-Tayeb *et al*., 2007; Robertson *et al*., 2006; Juge *et al*., 1998; Papagianni *et al*., 1999; Singh *et al*., 2011).

Enzymatic bioconversion of raw starch without prior gelatinisation has gained attention in both academia and industry because of the simplicity of the process and the fact that energy is saved. Raw starch-digesting amylases can catalyse the degradation of raw starch to reducing sugars and maltodextrins bypassing the gelatinisation step. *Aspergillus carbonarius* var (Bainier) Thom IMI 366159 isolated from rotten cassava in the laboratory of the University of Nigeria is able to produce large amounts of the raw starch-digesting enzyme, as well as the ability to digest a wide range of starch from different sources (Okolo *et al*., 2000). Other special features of the amylase from *A. carbonarius*, from a biotechnological perspective are their broad pH tolerance and stability, broad substrate specificity, production of maltose as the predominant degradation product and its temperature tolerance and stability (Okolo *et al*., 2000). These features differentiate the amylase of *A. carbonarius* from similar enzymes earlier reported and may provide practical advantages during large-scale industrial bioconversion of starch to value-added products.

It is well known that in submerged fermentations, agitation is important in order to achieve a good mixing, mass and heat transfer. Although mycelial damage at high stirrer speeds or power inputs can limit the acceptable range of speeds and subsequently, the oxygen transfers capability and the volumetric productivity of the fermentor (Amanullah *et al*., 1998). Agitation speed of culture broth has a variety of effects on microorganisms which include; damage to cell structure, morphological changes, as well as variations in growth rate and product formation (Papagianni *et al*., 1999). For each culture, optimum conditions of agitation exist that will partly depend on the resistance of the hyphae to mechanical forces and also on their physiological state (Mitchell and Lonsane, 1990; Paul *et al*., 1994; Moo-Young *et al*., 1992; Papagianni *et al*., 1998). Several authors (Lin *et al*., 2010; Ahamed and Vermette, 2010; Fadzilah and Mashitah, 2010; El-Enshasy *et al*., 2006; Park *et al*., 2002; Gerlech *et al*., 1998; Papagianni *et al*., 1998) have demonstrated the effects of mechanical forces on the morphology of filamentous microorganisms and the overall process productivities (Metz *et al*., 1981) in studies with *P. Chrysogenium* showed that the length of the mycelial particle decreased with increasing power input per unit mass in the reactor as the increased agitation caused the hyphae to become shorter, thicker and more highly branched. Studying the behaviour of three citric acid producing *A. niger* strains, Ujcova *et al*. (1980) observed that higher speed resulted in thicker, densely branched filaments while citric acid production was optimum within a narrow range of speeds and a drop in productivity resulted from higher stirrer speeds. The effect of agitation on *A. niger* was investigated by Papagianni *et al*. (1994, 1998) in a stirred tank and tubular loop bioreactor through a series of batch and feed batch experiments. Morphological measurements using image analysis showed that by increasing the intensity of agitation, the size of clumps decreased, as did the length of the filaments that arose from the core of the clumps while the diameter of the filaments increased, in both fermenters, specific rates of citric acid formation increased with agitation. Schugerl *et al*. (1998) and Gerlech *et al*. (1998) investigated the effect of different reactor types and types of shear stress (shake flask, stirred tank, airlift bioreactors) in studies with xylanase fermentation by

Aspergillus awomari. They observed that large loose hairy pellets were formed in pneumatically mixed reactors (airlift tower loop) while small and compact pellets were formed in shake flask and stirred tank reactors.

Ahamed and Vermette (2010) reported that influence of mechanical agitation using draft-tube airlift bioreactor, resulted in shorter mycelia hyphae and larger number of tips during the production of cellulase by *Trichoderma reesei*. The relationship between fungal morphology and process productivities aroused interest from both academia and industry and attempts have been made to manipulate morphology to achieve maximal performance. Despite all this studies there has been no report relating the production of amylase to the producing organism. Again the fact that RSDA is important in industrial application it will be interesting to document the effect of agitation on the morphology of *A. carbonarius* and raw starch digesting amylase production. Hence, considering the current economic situation and with emphasis on the use of locally available resources (bearing in mind that commercial enzymes are very expensive), the study focused on the use of readily available organism *Aspergillus carbonarius* to investigate how agitation speed will affect mycelia morphology, amylase enzyme activity, protein content and biomass formation during raw starch digesting amylase production in shake flask cultures. The results obtained are presented in this study.

MATERIALS AND METHODS

Microorganism: *Aspergillus carbonarius* var (Bainier) Thom IMI 366159 used in the study was obtained from University of Nigeria, Nsukka culture collection. The organism was maintained on Potato Dextrose Agar (PDA) slants subcultured every 3 months.

Inoculum and media preparations: *Aspergillus carbonarius* maintained on Potato Dextrose Agar (PDA) slants was transferred to fresh PDA plates and incubated at 30°C for 7 days. Spores were harvested from culture plates in sterile distilled water and spore suspensions were used as inocula at a level of 10^7 spores mL^{-1}. The fermentation medium comprised (g L^{-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. Five hundred millilitre (500 mL) flask containing 100 mL sterile fermentation medium was inoculated with spores and fermentation was carried out with rotary shaking, three levels of shaker speed were examined 100, 150 and 200 rpm and 30°C for 144 h.

Analytical methods: Dry weight was determined by filtering 10 mL of broth with pre-weighed Whatman No. 1 filter paper, washed with distilled water three times and dried in an oven at 80°C until a constant weight was obtained. Results were expressed as grams dry weight per litre of broth. Raw starch digesting amylase was assayed as described by Okolo *et al.* (2000). Total protein was estimated by Bradford (1976) method using Bovine Serum Albumin (BSA) as standard.

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 from 24 h twice a day for 144 h. Samples were immediately fixed with an equal volume of fixative as described by Packer and Thomas (1990) using 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 640-512 pixels. A magnification of 40x and 100x 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 and in some cases computer aided manual measurement was applied to measure and subtract false images. A total of 150 elements were analysed. These elements included clumps (dense aggregates of entangled hyphae) as well as freely dispersed form. Morphological parameters of interest for the freely dispersed mycelia were mean hyphal growth unit (total length divided by number of growing tips), mean total hyphal length and mean number of tips/mycelium. Clump/pellet morphology was quantified in terms of mean convex perimeter (Tucker *et al*., 1992).

RESULTS AND DISCUSSION

The effect of agitation speed on *Aspergillus carbonarius* morphology and RSDA production in shake flask cultures was investigated at 100, 150 and 200 rpm. Image analysis of broth samples at 100 rpm revealed, a mixture consisting mainly of pellets and a small number of entangled mycelia (clumps). At a speed of 150 or 200 rpm, the bulk of the mycelium was in the form of clumps (aggregated mycelial trees). Figure 1 shows the characteristic morphology of *A. carbonarius* at 144 h in shake flask cultures at different shaker speeds. At a shaker speed of 100 rpm pellets were large; hairy with a central core, formation of large pellets was partly due to low mechanical stress. Also, the clumps were loose with filaments that did not look fragmented (Fig. 1c and d). Small and compact pellets were formed at 150 and 200 rpm and clumps appeared more tightly packed. Papagianni *et al*. (1998) investigated the effect of agitation on *A. niger* morphology and citric acid production and they observed that the bulk of the mycelium was in the form of clumps. This is however, contrary to the results obtained in this study which showed that at a shaker speed of 100 rpm the bulk of the mycelium was in pelleted form, though the experiment was carried out in a shaker flask, in contrast to stirred tank used by Papagianni *et al*. (1998). It is not clear at present if the difference observed in the mycelia form or shape was caused by differences in the reactor systems used. In this regard while in this study the shaker method was used, Papagianni *et al*. (1998) used stirred tank method to obtain their results. Schugerl *et al*. (1998) and Gerlech *et al*. (1998) compared results obtained from shear stress and different reactor types; shake flask, stirred tank and airlift bioreactors in xylanase production by *A. awamori*., they observed that large, loose, hairy pellets were formed in mixed reactors (airlift tower loop) while small and compact pellets formed in shake flasks and stirred tank reactors. This is in agreement with the current results were small and compact pellets formed in shake flask cultures and confirms the fact that the method of agitation and reactor type will influence the shape of mycelium. Figure 2 shows the mean hyphal growth unit, mean number of tips/mycelium, mean total hyphal length and mean convex perimeter of clumps/pellets of *Aspergillus carbonarius* under different agitation speed. The results revealed that increasing the agitation speed caused a decrease in the mycelial particles examined. The mean number of tips per hyphal element (of freely dispersed form) was found to be similar at all three agitation speeds. Although it is not clear why this was the case, it is possible that the rate at which growth occurred and subsequent fragmentation of mycelia particles was relatively similar between

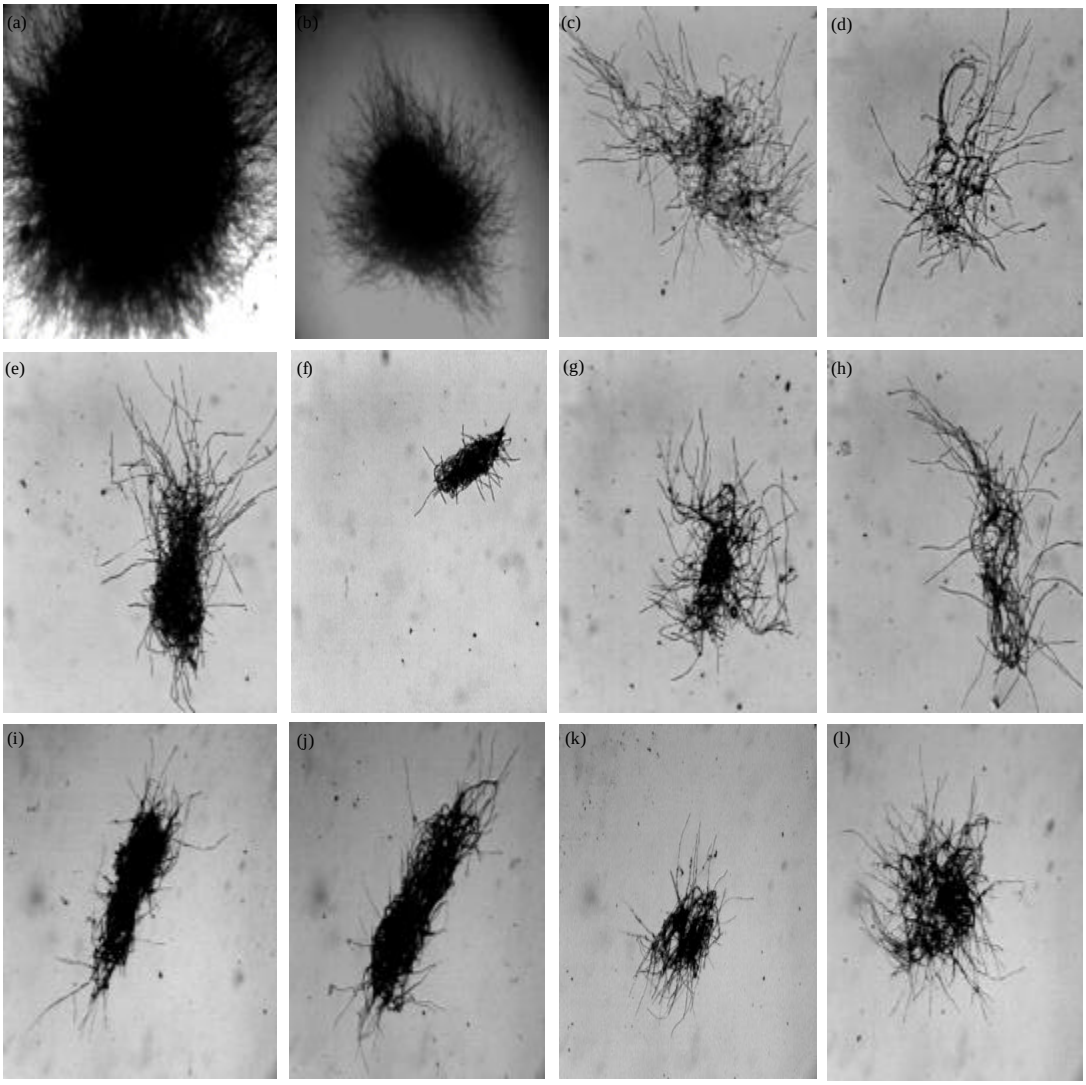

Fig. 1(a-l): Characteristic morphology of the fungus *Aspergillus carbonarius* in shake flask cultures at 144 h at different shaker speed, (a-d) Pellet/clumps at 100 rpm, (e-h) Pellets/clumps at 150 rpm and (i-l) Pellets/clumps at 200 rpm

the different agitation speeds giving rise to similar mean number of tips per mycelium or it may require a much higher agitation speed (say 500-1000 rpm) to obtain a remarkable difference in mean number of tip. Amanullah *et al.* (1999) reported similar values of (7.4±1.3 and 7.7±1.6) respectively for 550-700 rpm in chemostat cultures of recombinant *Aspergillus oryzae*. They proposed that the balance between growth and fragmentation were similar and hence, gave rise to similar mean number of tips per hypha. However, several authors have reported that hyphae are shorter, thicker and more highly branched at high agitation speeds compared to low speed (Dion *et al.*, 1954; Ujcova *et al.*, 1980; Metz *et al.*, 1981; Van Suijdam and Metz, 1981). The mean hyphal growth unit and the mean total hyphal length decreased as agitation speed increased.

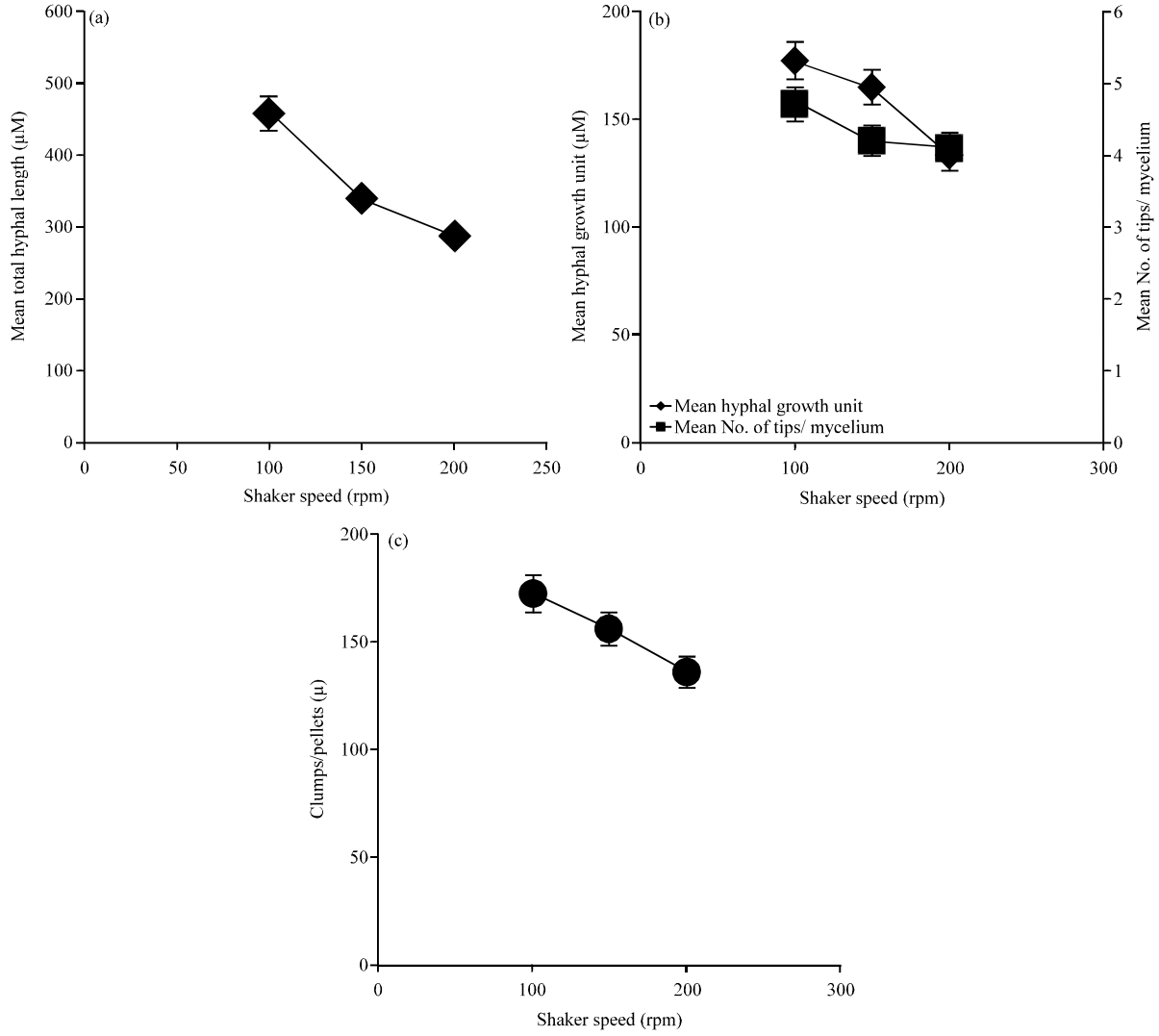

Fig. 2(a-c): The effect of shaker speed on morphology of *Aspergillus carbonarius*, (a) Mean total hyphal length, (b) Mean hyphal growth unit, mean number of tips/mycelium and (c) Mean convex perimeter of clumps/pellets at 144 h of shake flask cultures at different shaker speed (100,150 and 200 rpm)

Smith *et al.* (1990) and Metz *et al.* (1981) in their studies with *Pe. chrysogenum* showed that the length of mycelial particles decreased as agitation increased. The clump/pellet sizes (convex perimeter) decreased with increase in agitation speed. This observation is consistent with the results of other studies Papagianni *et al.* (1998), who investigated the relationship between the morphology of *Aspergillus niger* and citric acid production in a tubular loop and a conventional stirred tank bioreactor. Morphology characterized by the parameter P (mean convex perimeter of clumps) showed that increased agitation caused a reduction of clump sizes. On the contrary, Maazi *et al.* (1998) and Amanullah *et al.* (1999) reported that entangled mycelia may occur due to longer branches that have a higher probability of overlapping on each other. Thus, the mean projected area of branched mycelia became larger with time hence, under these conditions, this signify that mycelia growth dominated over mycelia fragmentation.

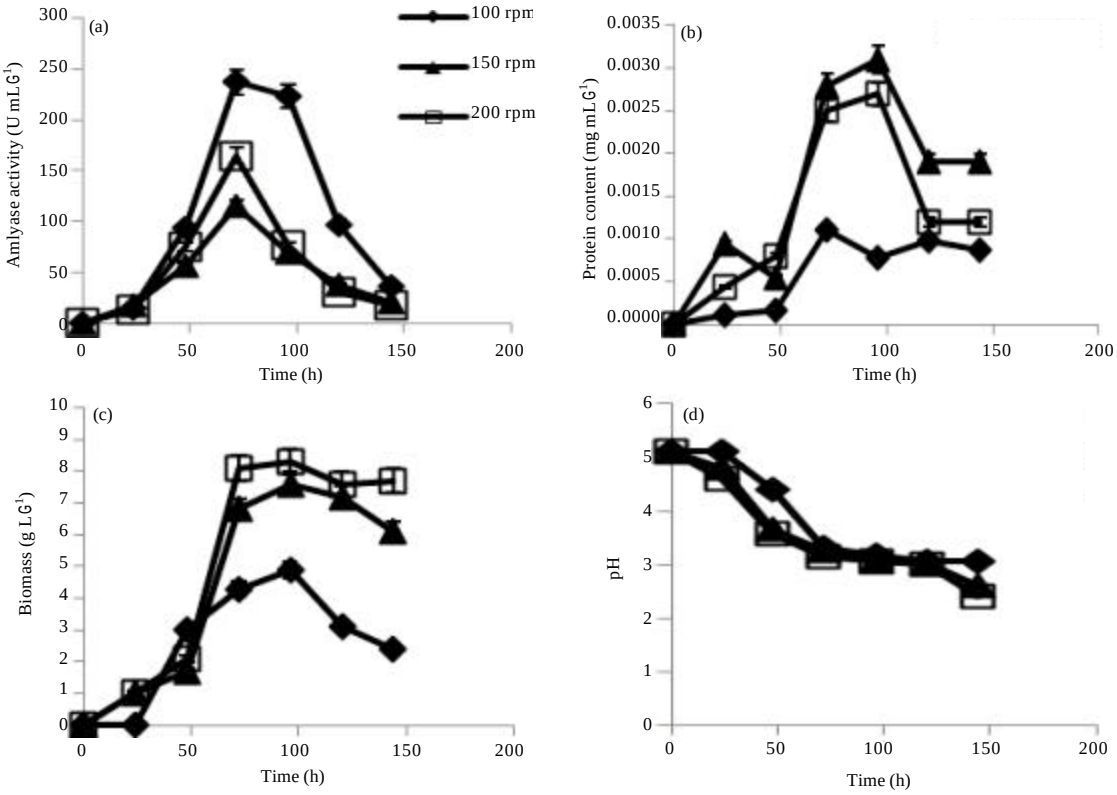

Fig. 3(a-d): Time course of (a) Raw starch digesting amylase activity, (b) Protein content, (c) Biomass concentrations and (d) pH, in shake flask cultures at different shaker speed (100, 150, 200 rpm)

The results in Fig. 3 show the different effects of agitation speed on the performance of *Aspergillus carbonarius* during raw starch digesting amylase production. The results in Fig. 3 show that the lower shaker speed of 100 rpm produced the highest RSDA activity (Fig. 3a). In contrast, a speed of 150 rpm produced the least enzyme activity. Generally peak production of RSDA was achieved at 72 h at all shaker speed studied. In contrast to the results found in RSDA activity, agitation speed of 150 rpm released the highest protein content while a shaker speed of 100 rpm released the least protein content (Fig. 3b). These results (Fig. 3a, b) confirm the well known fact that all enzymes are protein but not all proteins are enzymes. In these results (Fig 3a and b) agitation speed of 200 rpm produced intermediate results observed for agitation speed of 100 and 150 rpm. It is interesting to observe that while agitation speed of 200 rpm produced intermediate results for RSDA and protein content when compared to agitation speed of 100 and 150 rpm, higher biomass production was achieved at agitation speed of 200 rpm (Fig. 3c).The exact reasons for these observations are not clear at present. The results in Fig. 3d show that agitation speed did not affect the pH of the medium during amylase production from *Aspergillus carbonarius*.

Many investigators have discussed effect of agitation on morphology and biosynthesis. There are cases where growth rate and productivities were linked to morphology or cases were no link was observed (Markel and Bronnemeier, 1985; Belmer-Beiny and Thomas, 1991; Anusha *et al*., 2012).

From the trends of the RSDA time courses, it is very likely that fungal morphology played a key role in the process (Amadi and Okolo, 2012). In the present study, the three different agitation levels resulted in the development of distinctive morphological forms. Pellets predominated at 100 rpm shaker speed which resulted in increased RSDA levels and this morphological form has been associated with increased specific growth rates. Formation of mycelial pellets is often regarded as a prerequisite for successful production of microbial metabolites, including some fungal enzymes such as polygalacturonidase or glucosidase and RSDA (Hermersdorfer *et al*., 1987; Amadi and Okolo, 2012). But in some cases, the free filamentous morphology is preferred for optimal metabolite formation (Schugerl *et al*., 1998). In any case, Polygalacturonidase synthesis correlates well with the pelleted morphological type of *A. niger*. The more compact the pellet, the greater the polygalacturonidase synthesis, regardless the composition of the medium (Hermersdorfer *et al*., 1987). In the present study, the increased rates of RSDA production from pellets may suggest that this morphological form is most suitable for the RSDA formation (Amadi and Okolo, 2012). However, as studies have shown the centre of the pellets becomes autolysed with time and does not contribute to metabolite biosynthesis anymore (Wittler *et al*., 1986). Therefore, a lower proportion of the biomass is involved in biosynthesis and this may explain the reversing trend observed beyond 75 h of fermentation. This is not the case with the bulk of clumped morphology obtained from 150 and 200 rpm which had lower RSDA levels and higher biomass concentration. The high biomass concentration could have been the reason for low volumetric enzyme productivity in terms of quantity with the increased agitation speed (150 and 200 rpm). Lower productivities have been reported at high agitation speeds and were attributed to cell damage (Smith *et al*., 1990). Paul *et al*. (1999) also reported that citric acid yield was decreased with high agitation speed in submerged cultures of *Aspergillus niger*.

In this study, fragmentation is likely to have occurred at increased agitation levels resulting in low levels of RSDA production and high biomass concentration in 150 and 200 rpm. Usually fragmentation resulted from increased shear stress applied to the mycelium which is characteristic of high stirrer speed (Ayazi Shamlou *et al*., 1994). Fragmentation of hyphal elements and pellets during submerged fermentation often results in growth renewal since fragments may act as centres for new growth, enabling reseeding of the mycelia population (Papagianni, 2004). Long and ramified hyphae increase the number of possible interaction sites with the substrate (De Nicolas-Santiago *et al*., 2006). The number of newly growing tips from the main hyphae is an indication of active biomass accumulation in the culture medium. Protein secretion in filamentous fungi is believed to mainly occur at the tips of growing hyphae because these tips are more porous, therefore, facilitating exo-enzymes to pass through the cell wall (Peberdy, 1994; Wosten *et al*., 1991; Wessels, 1993). Thus, factors that increase the number of active tips may improve protein yield (Juge *et al*., 1998). Branched hyphae resulted in an increase in the number of tips and yielded more proteins into the fermentation broth (Wosten *et al*., 1991). This may account for the increase in protein content and increased biomass concentration at 150 and 200 rpm earlier observed. Tip growth and branching result in different macroscopic appearances of the culture, ranging from single hypha elements, dispersed mycelia, over connected networks of hyphae up to distinct biomass particles. Previous studies (Peberdy 1994; Juge *et al*., 1998; Ahamed and Vermette, 2009) have shown that the morphology and physiology of filamentous microorganisms in submerged cultures are dependent on culture conditions. Ahamed and Vermette (2010) reported that in fed-batch cultures, *T. reesei* were mainly found in the dispersed form which comprises

unbranched, branched and entangled mycelia. In the filamentous growth form a lower proportion of cells become autolysed with time and this can result in increased final productivities compared with the pelleted growth form (Papagianni *et al*., 2001).

CONCLUSION

In this study, from the view point of using locally available materials authors have also been able to demonstrate that *Aspergillus carbonarius* morphology is also affected by the agitation speed during RSDA production. At lower agitation speed pellet morphology dominated resulting in higher RSDA activity. But increased protein production was observed at the highest stirrer speed. In other words, when studying enzyme stabilization it will be wise to use pellet morphology to obtain enzyme with high activity.

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