

Induced; thermal influence on morphology of *Aspergillus carbonarius* during raw starch digesting amylase production

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ABSTRACT

Aim: To investigate increased thermal influence on morphology of *Aspergillus carbonarius* during RSDA production.

Place and Duration of Study: Microbial fermentation Unit, Department of Microbiology, Faculty of Biological sciences, University of Nigeria, Nsukka between July 2009 and August 2010.

Methodology: In shake flask cultures thermal influence on *A. carbonarius* morphology and productivity investigated. Mycelial morphology was characterised by means of image analysis using as parameters, mean diameter, roughness, circularity and compactness of pellet. Thermal effect on amylase activity, total protein, biomass concentration and pH were also investigated.

Results: Shifting the temperature from 27°C to 37°C significantly affected the morphological parameters of the pellets, but RSDA activity was not altered. The interesting thing about the morphology is the shearing off of the hairy part of the pellet at an increased temperature and subsequent agglomeration. At 27°C the RSDA activity increased steadily with an optimum activity of 293U/ml at 96h and subsequently decreased to 75U/ml by the end of the fermentation. At 37°C a maximum activity of 291U/ml was achieved at 72h of fermentation but this decreased to 87U/ml at the end of fermentation. Higher biomass concentration and total protein were obtained at 37°C. The pH dropped from an initial of 5.0 to 3.0 and 2.5 for 27°C and 37°C temperature conditions respectively.

Conclusion: Induced thermal increase resulted to changes in pellet morphology but raw starch digesting amylase activity was not altered.

Keywords: Fungal morphology, *Aspergillus carbonarius*, RSDA, Pellets, Thermal effects

1. INTRODUCTION

Filamentous fungi are interesting organisms because of the inherent beauty of their fruiting bodies as well as their complicated and scientifically remarkable behavior (Kossen, 2000). They have the ability to produce a variety of useful, commercially valuable products such as organic acids, antibiotics, enzymes etc (Cao *et al.*, 1996, Cases Lopez *et al.*, 2004, Papagianni 2004 and Steel 1954.). These fungi often grown in submerged cultures exhibit different morphological forms; suspended mycelia, clumps or pellets (Metz and Kossen 1977 and Papagianni 2004). However the pellet form favors most fungal fermentation in the sense that it makes repeated batch fungal fermentation possible and rheological

problems becomes less because there are better mass and oxygen transfer into biomass and lower energy consumption for aeration and agitation (van Suidjam *et al.*, 1980).

Changes in fungal fermentation are influenced by lots of factors such as, medium composition, pH, agitation, dissolved oxygen, reactor type and scale, inoculum size and temperature (Ahamed and Vemette 2010, Amadi and Okolo 2012, Amanullah *et al.*, 2002, Calsen *et al.*, 1996, Cui *et al.*, 1998, Johansen *et al.*, 1998, Papagianni and Moo Young 2002, Papagianni *et al.*, 2006 and Schugerl *et al.*, 1998).

Despite all the studies on morphology and metabolite production relatively little has been published on temperature effects on growth and metabolite production with respect to filamentous organisms. Although temperature is an environmental parameter and can be controlled with ease, changes in temperature produce simultaneous changes in culture variables. Temperature influences enzyme activity and therefore microbial metabolism. Both enzyme productivity and also the composition of the enzyme mixture produced by the fungi depend on temperature. Dissolved oxygen tension is also temperature dependant and varies inversely with increasing temperature. Consequently, nutritional and pH requirements for growth may be influenced by the temperature. Madigan *et al.*, (2000) observed temperature sensitivity of the protoplasmic membrane in the presence of metabolizable carbon substrates to be an important determinant of maximum growth temperature. The influence of cultivation temperature on growth kinetics of *As. oryzae* was investigated by Carlsen *et al.*, (1995) in a series of batch experiments. The specific growth rate of the fungus, grown in the form of pellets with approximately the same size distribution, increased as temperature rose from 27 to 35°C to reach its optimum at 35°C. Temperature also affects pellet formation. Braun and Vecht-Lifshitz (1991) suggested reduction in temperature, among other possible approaches, for inducing mycelial pellets formation in fermentation cultures which also agrees with the study of Schugel *et al.*, (1998) on the production of xylanase by *As. awamori* in shake flask, studies at constant rotation speed revealed that cell volume and the amounts of various morphological forms were determined by temperature in the range of 25-35°C. Calsen *et al.*, (1995) investigated temperatures of 27 to 40°C at pH above 4.0 and with inoculum consisting of spores they observed that pellets were formed and the pellet size distribution was found to be temperature dependent.

Although a number of reports exist on *Aspergillus sp* and metabolite production and morphology little is known about *Aspergillus carbonarius* particularly with respect to RSDA production. *Aspergillus carbonarius* var (Brainer) Thom IMI 366159 was isolated in the laboratory of University of Nigeria (Okolo *et al.*, 2000) and it produces large amount of raw starch digesting amylase. Raw starch digesting amylase has earned industrial interest as it hydrolyses raw starch straight to simple sugars without heating, saving energy cost. More so having an enzyme that has the ability to withstand increased temperature is also very relevant. In view of this induced, increased thermal influence on morphology of *Aspergillus carbonarius* in shake flask cultures was investigated.

2. MATERIAL AND METHODS

2.1 Microorganisms, inoculum and media preparations.

Aspergillus carbonarius var (Brainer) Thom IMI 366159 (University of Nigeria, Nsukka) was used in this study. The organism was maintained on Potato Dextrose Agar (PDA) slants subcultured every 3 months. *Aspergillus carbonarius* maintained on PDA slants was transferred to fresh PDA plates and incubated at 30°C for 7 days. The fermentation medium comprised (g litre⁻¹): Raw cassava starch (*Manihot utilissima* Crantz), 20; yeast extract, 5; KH₂PO₄, 7; CaCl₂·6H₂O, 0.3; and MgSO₄·7H₂O, 0.3 in distilled water and autoclaved at 121°C for 15 min at 15 psi.

2.2 Culture conditions (shake flask cultures)

Spores were harvested from culture plates in sterile distilled water and spore suspensions were used as inocula at a level of 10⁷ spores ml⁻¹. Five hundred millilitre (500mL) flask containing 100ml fermentation medium was inoculated with spores and cultivation took place at either 27°C or 37°C on a rotary shaker at 100 rpm for 144h.

2.3 Analytical methods

Dry weight was determined by filtering 10ml of broth with preweighed Whatman No. 1 filter paper, washing with distilled water three times and drying 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). In brief, RSDA assay was performed by mixing 0.5 ml 1% (w/v) of raw cassava starch in 0.1M 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 were estimated by DNS method of Millar. One unit of amylase was defined as the amount of enzyme that produced reducing sugar equivalent to 1 µmol of glucose from raw cassava starch per min under the assay conditions. Total protein was estimated by Bradford (1976) method using bovine serum albumin (BSA) as standard.

2.4 Image analysis and processing

Image capture was carried out via a semiautomatic image analyser Moticam 1000 digital microscope camera UK, (Motic Images Plus 2.0ML) with a USB 2.0 cable. Mounted on a microscope (Optika, Italy) and connected to a PC. Samples for morphological characterization were taken from 24h twice a day for 144h. Samples were immediately fixed with an equal volume of fixative as described by Parker and Thomas (1990), using 13 ml of 40% w/v formaldehyde and 5ml glacial acetic acid added to 200 ml of 50% w/v ethanol. Images were captured at 640-512 pixels. A magnification of X100 was applied for measurements on mycelia particles.

Medium particles, artifacts, debris and morphological particles which were touching the organism and could have biased the results were removed. For each sample, the morphology of 50 pellets was characterized by measuring the area and perimeter of the pellets. The morphology of the pellets was characterized by their mean diameter, circularity, roughness and compactness. The circularity factor was estimated by the ratio of Feret's minimum diameter to Feret's maximum diameter of the pellets, compactness was estimated as the ratio of the projected area of the hyphae in the clump to the projected convex area of that clump, the latter being the area after filling internal voids and concavities in the clumps external perimeter. Roughness was given by the circularity factor $C = \text{perimeter}^2 / 4\pi \times \text{area}$ (Paul and Thomas 1998 and Tucker *et al* 1992)

3. RESULTS AND DISCUSSION

3.1 Pellet Morphology

Pellet morphologies have been classified into three loosely banded groups encompassing the variations observed under different fermentation regimes. Members of the first group have a compact central core with a fluffy (hairy), loosely packed filamentous, outer zone. The next group shows a compacted core that is smooth with limited lateral growth. Pellets of the final group have developed from the previous two forms and have a hollow core (Clark D.S. 1962 and Steel *et al.*, 1954). Pellets morphology has also been characterized using various parameters, which include hyphal length, size and shape of the pellet (Cui *et al.*, 1998, Olsvik *et al.*, 1993 and Riley *et al.*, 2000). In the present study pellets were differentiated from the clumps by their level of grayness. The amount of grayness used as critical threshold was predetermined by analyzing many images. The cells formed mainly pellets irrespective of the cultivation temperature; Gelech *et al.*, (1998) also observed that at 25°C pellets dominated, at 30°C pellets formed but were decomposed at 50h. Reason for pellet dominance may be due to the initial pH value of 5.0. The phenomenon of pellet formation is been reported to be greatly influenced by the pH, and the tendency of the mycelium to form pellets increases as the pH value of the culture raises (Whitaker and Long 1973, Braun and Vecht-Lifshitz 1991 and Gerlech *et al.*, 1998). The present result is in agreement with Carlsen *et al.*, (1995) who observed in a batch cultivation of *As. oryzae* in a stirred tank reactor with an inoculum consisting of 4×10^8 spore/ml that at pH values below 2.5, the mycelium grew heavily vacuolated and the cells appeared swollen resulting in poor growth, at pH value 3.0-3.5 freely dispersed hyphal elements resulted, while at pH 4.0-5.0, the broth consisted of both pellets and freely dispersed hyphae, pellet formation was caused by agglomeration of the spores at pH values above 4.0. Similar were the observation of Steel *et al.*, (1954) that filamentous growth for *As. niger* occurred at pH values below 5.0. In addition formation of large pellets was partly due to low mechanical stress (Amadi and Okolo 2012, Gelech *et al.*, 1998.).

3.2 Thermal influence on pellet morphology

Figure 1 (a,b,c) shows pellets from a submerged cultivation of raw starch digesting amylase producing *Aspergillus carbonarius* at 27°C. The pellets morphology expressed different forms, from the spherical to oval and intermediates, like the elongated irregular pellets which are often seen as crossover morphology. Pellets were also observed to possess a central core of densely parked hypha surrounded by hairy region containing radially growing hypha. These features are characteristic of pellet morphology during filamentous growth. Braun and Vecht-Lifshitz (1991) classified pellets as spherical or oval during filamentous growth Wucherpfennig *et al.*, (2011) observed all pellet morphology including the elongated form in their study on the effect of osmolarity on *Aspergillus niger* morphology and productivity.

It would appear from the results in Fig 2, where submerged cultivation of raw starch digesting amylase producing *Aspergillus carbonarius* was carried out at a higher temperature (37°C) there was reduction in dense packing of hypha. Under this submerged cultivation of raw starch digesting amylase enzymes from *Aspergillus carbonarius*, pellets structures were fluffy with much loose outer zone as shown in Figure 2 (b and c) or fluffy with compact centre and approximately the same size as shown in Fig. 2 (a, c and e). In this regard submerged cultivation temperature may have affected pellets structures (Figs 1 and 2).

Figure 3 (a,b,c,d,e) shows sheared off hairy parts of *Aspergillus carbonarius* pellets from a submerged raw starch digesting amylase fermentation at 37°C. Many authors have described mycelia fragmentation as a result of mechanical agitation (Amanullah *et al.*, 1999, Johansen *et al.*, 1995, Li *et al.*, 2002, Metz *et al.*, 1981, Papagianni *et al.*, 1995, 1998, 1999, 2002, and Ujcova *et al.*, 1980,) and increased vacuolation levels (Papagianni *et al.*, 1999, Paul *et al.*, 1994). The former is not in agreement with our result as the fermentation was carried out at a low agitation speed of 100 rpm. Hyphal vacuolation is a natural process of aging which may weaken the filaments and expose them to damage, conditions such as glucose supply at the maintenance rate or oxygen limitation may contribute to natural process of vacuolation and also results in increased fragmentation Havey *et al* (1998). Hence the shearing off of the hyphae or fragmentation may be due to acceleration of ageing process from increased temperature. Also the pH of the fermentation medium (3.0) could have influenced the shaving off of the hairy outer zone since dispersed mycelia resulted at pH 3.0-3.5 as reported by Carlsen *et al* (1995). The strong temperature effect on morphology may also be probably due to oxygen limitation (Henzler and Schedel 1991). Gelech *et al* (1998) reported that at 25°C the dissolved oxygen concentration was high enough for the pellets. With an increase of temperature from 25°C to 30°C and 35°C respectively, the metabolic activity of the cell increased and the solubility of oxygen in the liquid medium decreased, and growth became oxygen transfer limited therefore with increasing cell concentration and oxygen limitation, pellets were decomposed and filamentous mycelium was formed. In the present study however hyphal fragmentation or shaving off of the hairy part of the pellets may also be as a result of depletion of dissolved oxygen in the pellet core and subsequently faster pellet autolysis.

Fig 4 (a,b,c) shows sheared off hairs from the surface of the pellets (at 37°C) coming together. The morphological properties of filamentous fungi play a significant role in metabolism during fermentation process (Nielson *et al* 1995, Johansen *et al* 1998, Paul *et al* 1999). However, the reason behind the coming together of the shaved off hairs is not clear at present. It is possible that agglomeration observed is likely to be as a result of swirling speed of (100 rpm) applied.

Figure 5 (a,b,c,d) shows the mean diameter, circularity, roughness and compactness of the pellets during the submerged cultures of *Aspergillus carbonarius* at varying temperature. In general, the results in Figure 5 (a, b, c, and d) show that mycelia growth occurred at both temperatures of 27°C and 37°C. These results in terms of mycelial growth agree with other studies (Alam *et al.*, 2001; Eng *et al.*, 2003; Saha *et al.*, 2008). However, there was significant variation in the morphological parameter between the pellets grown at these different temperature conditions. At both temperatures of 27°C and 37°C, the circularity (Figure 5a), compactness (Figure 5b) mean diameter (Figure 5c) and roughness of pellets (Figure 5d) all increased continuously throughout the fermentation, with higher results being obtained at 27°C. The effect of temperature was more pronounced on compactness of pellets (Figure 5b) where maximum results occurred after 120h and leveled off when fermentation was performed at 27°C but significantly dropped off when fermentation was carried out at 37°C. As fermentation progressed, the outer hairy region of the mycelia pellets formed at 37°C sheared off, hence reducing the core area with subsequent decrease in pellet diameter and compactness. Again, with increase in temperature, the metabolic activity increased and the oxygen solubility decreased. These can result in limitation of growth in the shake flask with subsequent decomposition of the pellets. In this regard, pellet breakup at 37°C was due partly to oxygen limitation and partly due to decrease in pH values. On the other hand overall change that resulted in the morphology of *Aspergillus carbonarius* by the shift in temperature from 27°C to 37°C can be explained biochemically using the mechanism that occurs in dimorphic fungi. Dimorphic fungi are known to express phase transition due to numerous factors. External stimuli change intracellular signaling, metabolism and gene expression in dimorphic fungi triggering phase transition, other stimuli such as oxidative stress, changes in carbon dioxide tension and steroid hormones also regulate morphogenesis. In spite of all these temperature change is a major environmental stimulus that triggers (morphogenesis) the phase transition from mould (25°C) to yeast (37°C). Although these factors are associated with dimorphic fungi the same mechanism may apply for the resulting changes in pellet morphology under induced increase in temperature in the present study. Further more changes in membrane fluidity and lipid composition have been said to contribute to morphological transition for instance when cells adapt to changes particularly temperature multiple changes occur in the lipid composition of the plasma membrane leading to remodeling and reorganization in the membrane (Maresca and Kabayashi 2000, Gauthier and Klein 2008) lower temperatures decrease membrane fluidity, the reverse being the case for higher temperature. This may eventually bring about change in cell shape and cell wall composition and may also account for the high compact nature of pellets at 27°C and fluffy nature of pellets at 37°C. The histidine kinase two component system have been shown to play a role in environmental sensing and cell development in eukaryote (Dickman and Yarden 1999)

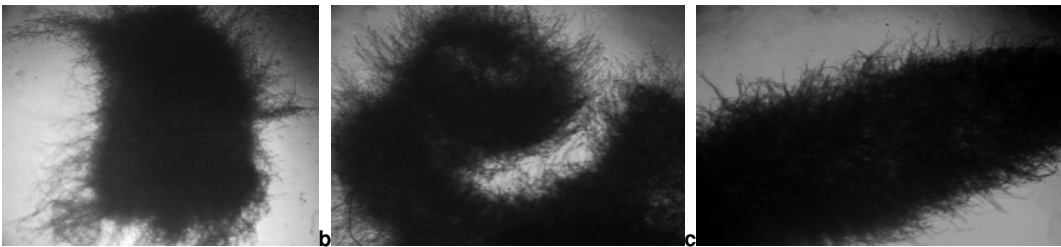


Fig. 1 (a, b, c) *Aspergillus carbonarius* pellets from a submerged raw starch digesting amylase fermentation at 27°C

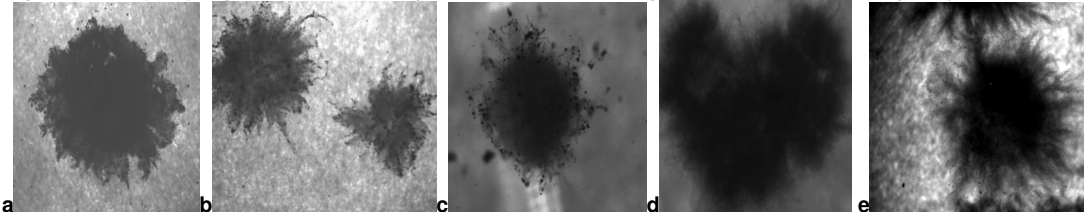


Fig. 2 (a,b,c,d,e) *Aspergillus carbonarius* pellets from a submerged raw starch digesting amylase fermentation at 37°C.

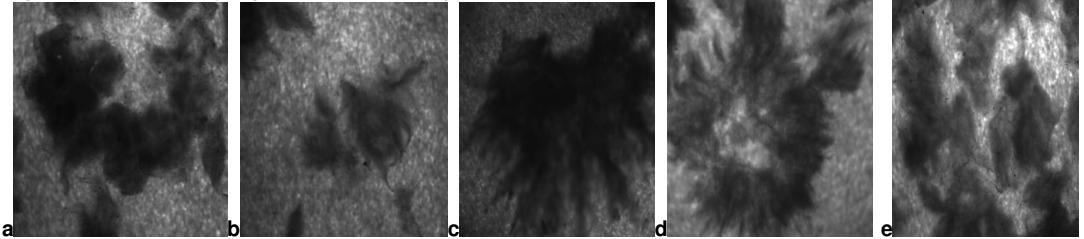


Fig.3 (a,b,c,d,e) sheared off hairy parts of *Aspergillus carbonarius* pellets from a submerged raw starch digesting amylase fermentation at 37°C.

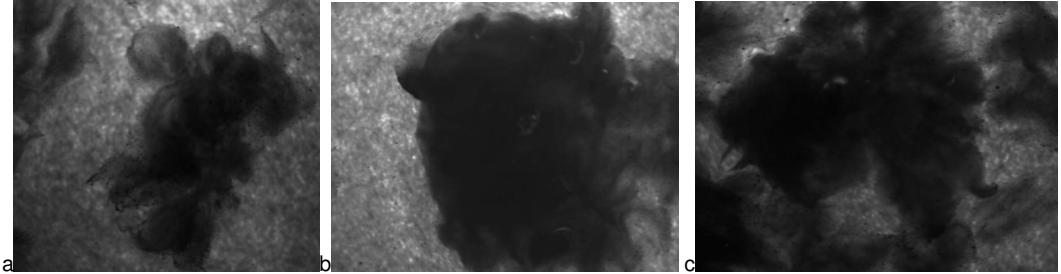
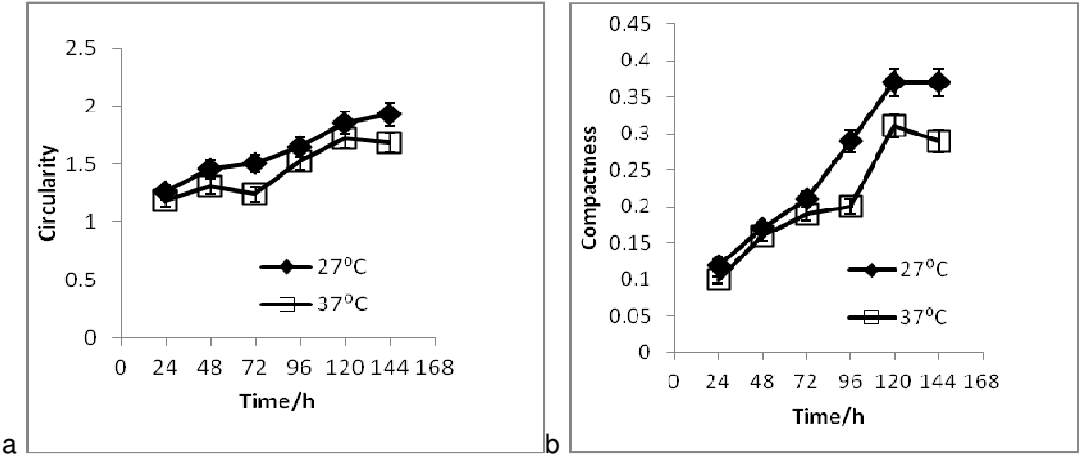


Fig 4 (a,b,c) sheared off hairs (at 37°C) agglomerating



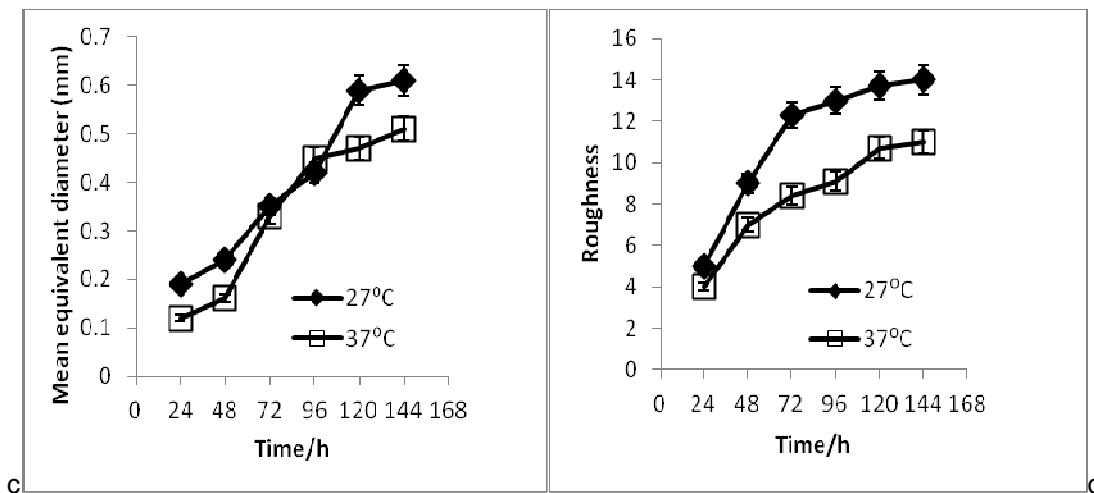


Fig.5 circularity (a), compactness (b), mean equivalent diameter (c), roughness (d) of *Aspergillus carbonarius* in shake flask cultures at different temperatures (27°C. And 37°C)

3.3 Thermal influence on culture variables

Figure 6 shows the time profile of thermal influence on culture variables - RSDA activity (Figure 6a), total protein (Figure 6b), biomass concentration (Figure 6c) and pH (Figure 6d) at the two temperature conditions of 27°C and 37°C studied. At both temperatures, RSDA activity increased steadily, but with an early peak of (72 h) reaching a maximum value of 291 U/mL when fermentation was performed at the higher temperature of 37°C, some studies have shown increase in temperature influencing product yield by organism (Snajdr and Baldrian 2007, Urek and Paralioglu 2007). In contrast when fermentation was carried out at 27°C marginal higher RSDA activity (293 U/mL) was achieved after 96 h. Overall, an increase in temperature from 27°C to 37°C did not show significant increase in RSDA activity (Figure 6a), suggesting that temperature will have minimal effect in RSDA production. In contrast to the observation for RSDA production, protein content was higher at 37°C than at 27°C (Figure 6b). These observations are interesting because they show that not all the proteins found in the medium (Figure 6b) can produce amylase (Figure 6a). These observations (Figures 6a and 6b) could also be responsible for the observation made for biomass (Figure 6c) because the fermentation medium (37°C) had the higher biomass (Figure 6c). The pH of the medium dropped from 5.12 to 3.0 at 27°C by the 72h of fermentation and remained relatively constant (Figure 6d). The drop in pH was however, greater when fermentation was carried out at 37°C rather than at 27°C.

Authors had earlier observed that increased temperature 37°C resulted in pellet breakup. This may account for increase in protein and biomass concentration. Studies have shown that protein secretion in filamentous fungi mainly occur at the tip of growing hyphae because the tips are more porous they facilitate exo-enzymes to pass through the cell wall (Peberdy 1994, Wosten *et al* 1991 and Wessels 1993). Hence factors that increase the number of active tips may improve protein yield into fermentation broth (Judge *et al* 1998 and Wosten *et al* 1991). Again, Papagianni (2004) stated that fragmentation of hyphal elements and pellets during submerged fermentations often results in growth renewal as

fragments may act as centers for new growth, enabling reseedling of the pellet populations. Furthermore the number of newly growing tips from hyphae is an indication of active biomass accumulation in the culture medium.

From the authors microbiology point of view, from this study, the fact that RSDA activity was not reasonably altered in both 27°C and 37°C authors suggest that protein kinases which mediate the phosphorylation that regulates protein function directly, or via signal transduction, in many areas of cell metabolism (McIntyre et al 2001) may be responsible, as they have been linked to signal transduction cascade, protein modification and fungal morphogenesis. In filamentous fungi and in eukaryotes the protein kinases that phosphorylate either serine or threonine (Ser/Thr kinases) are mostly the cAMP-dependent kinases (PKA), protein kinases type C (PKC), mitogen-activated kinases (MAP), and p21-activated kinases (PAK) (Dickman and Yarden 1999). Of all these the PKAs are said to play important role in fungal development (McIntyre *et al.*, 2001). Since the PKAs play important role in fungal development and morphology is closely linked to productivity they may be responsible for the change in the pellet morphology while still enabling the fungi retain their ability to secrete enzymes which brought about the unaltered enzyme activity in the different temperature (27°C to 37°C) conditions.

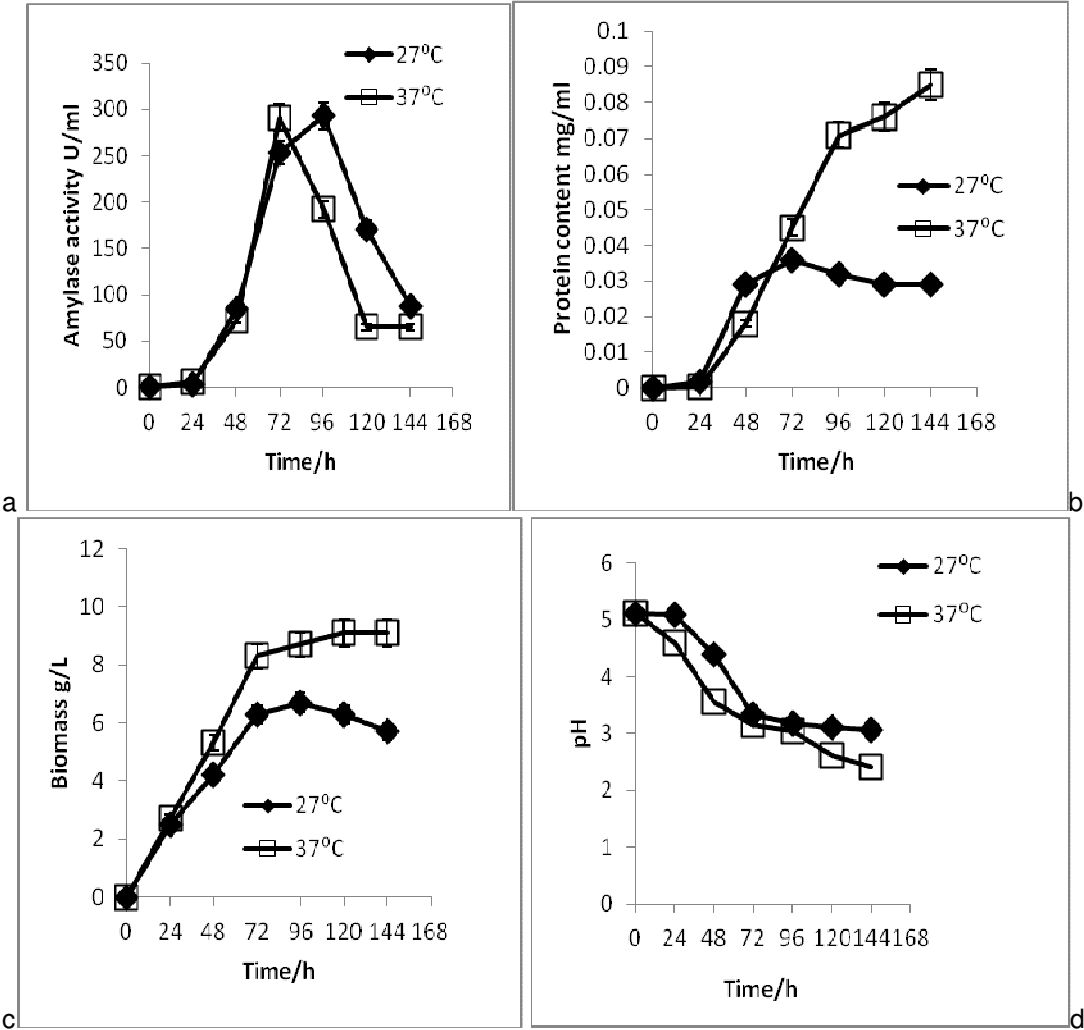


Fig. 6 Time course of RSDA activity (a), Protein content (b), and Biomass concentration (c) pH (d) in shake flask cultures of *Aspergillus carbonarius* at different temperatures 27°C and 37°C

4. CONCLUSION

Results of this study revealed that increase in temperature, induced changes in pellet morphology, produced simultaneous changes in other culture variables but RSDA activity was not altered.

COMPETING INTERESTS

Authors have declared that no competing interest exists.

AUTHORS' CONTRIBUTIONS

B.N. Okolo supervised the study. O.C. Amadi and B. N. Okolo designed and wrote the protocol. O.C. Amadi managed the analyses of the study and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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