

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 behaviour (Kossen 2000). They have the ability to produce large 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 are often grown in submerged cultures exhibiting different morphological forms; suspended mycelia, clumps or pellets (Metz and Kossen 1977, Papagianni 2004). However the

pellet form favours 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 shape and temperature (Schugerl *et al* 1998, Johansen *et al* 1998, Papagianni *et al* 2006, Papagianni and Moo Young 2002, Cui *et al* 1998, Calsen *et al* 1996, Amadi and Okolo 2012, Amanullah *et al* 2002).

Despite all the studies on morphology and metabolite production relatively little has been published on the 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). 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, artefacts, 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 characterised 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 $C = \text{perimeter}^2 / 4\pi \times \text{area}$ (Tucker *et al* 1992 and Paul and Thomas 1998)

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 have also been characterized using various parameters, which include hyphal length, size and shape of the pellet (Olsvik *et al* 1993, Cui *et al* 1998, 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, 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 (Gelech *et al* 1998, Amadi and Okolo 2012).

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 were larger and have a compact central core with fluffy (hairy) loosely packed filamentous, outer zone.

Figure 2 (a,b,c,d,e) shows the pellets from a submerged culture of raw starch digesting amylase producing *Aspergillus carbonarius* at 37°C. Pellets were approximately of the same size, bulkier, fluffy with dense outer zone, which were shaved off during fermentation as shown in (Figure 3).

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 (Metz *et al* 1981, Ujcova *et al* 1980, Johansen *et al* 1995, Papagianni *et al* 1995, 1998, 1999, Amanullah *et al* 1999, 2002 and Li *et al* 2002) and increased vacuolation levels (Paul *et al* 1994, Papagianni *et al* 1999). The former is not in agreement with our result as the fermentation was carried out at a low agitation speed of 100rpm. However 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 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 leading to autolysis. 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)

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. There was significant variation in the morphological parameter between the pellets grown at different temperature conditions. Mean diameter and roughness of pellets increased continuously throughout the fermentation, were pellet size and roughness indicated lower values with increase in temperature. Circularity and compactness of pellets decreased at later stage of fermentation. As fermentation proceeded, the outer hairy region of the mycelia pellets formed at 30°C sheared off, hence reducing the core area with subsequent decrease in pellet diameter and compactness. With increase in temperature, the metabolic activity increased and the oxygen solubility decreased. This caused growth limitation in the shake flask and subsequently the decomposition of the pellets. We suggest that pellet breakup at 37°C was due partly to oxygen limitation and partly due to decrease in pH values.

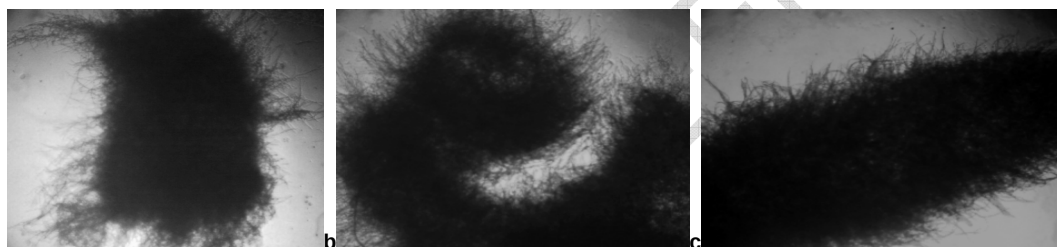


Fig. 1 (a, b, c) *Aspergillus carbonarius* pellets from a submerged raw starch digesting amylase fermentation at 27°C

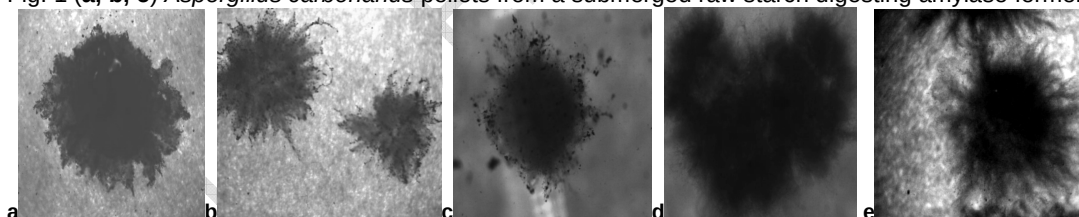


Fig. 2 (a,b,c,d,e) *Aspergillus carbonarius* pellets from a submerged raw starch digesting amylase fermentation at 37°C.

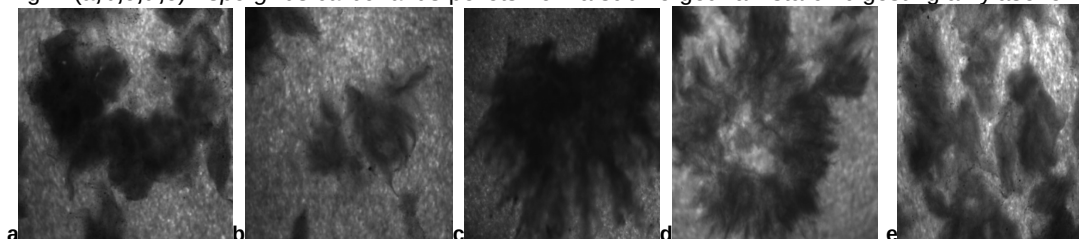


Fig.3 (a,b,c,d,e) sheared off hairy parts of *Aspergillus carbonarius* pellets from a submerged raw starch digesting amylase fermentation at 37°C.

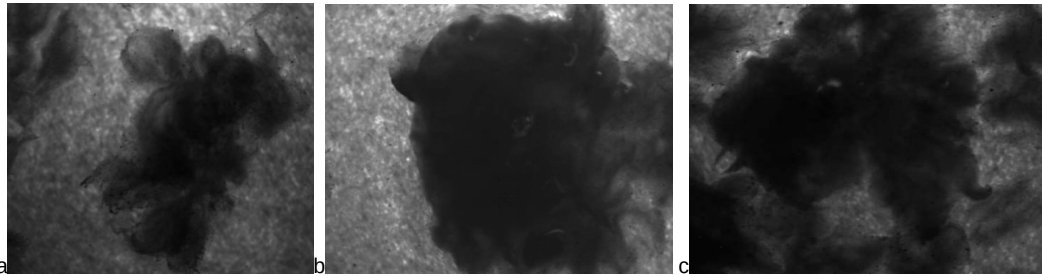


Fig 4 (a,b,c) sheared off hairs (at 37°C) agglomerating

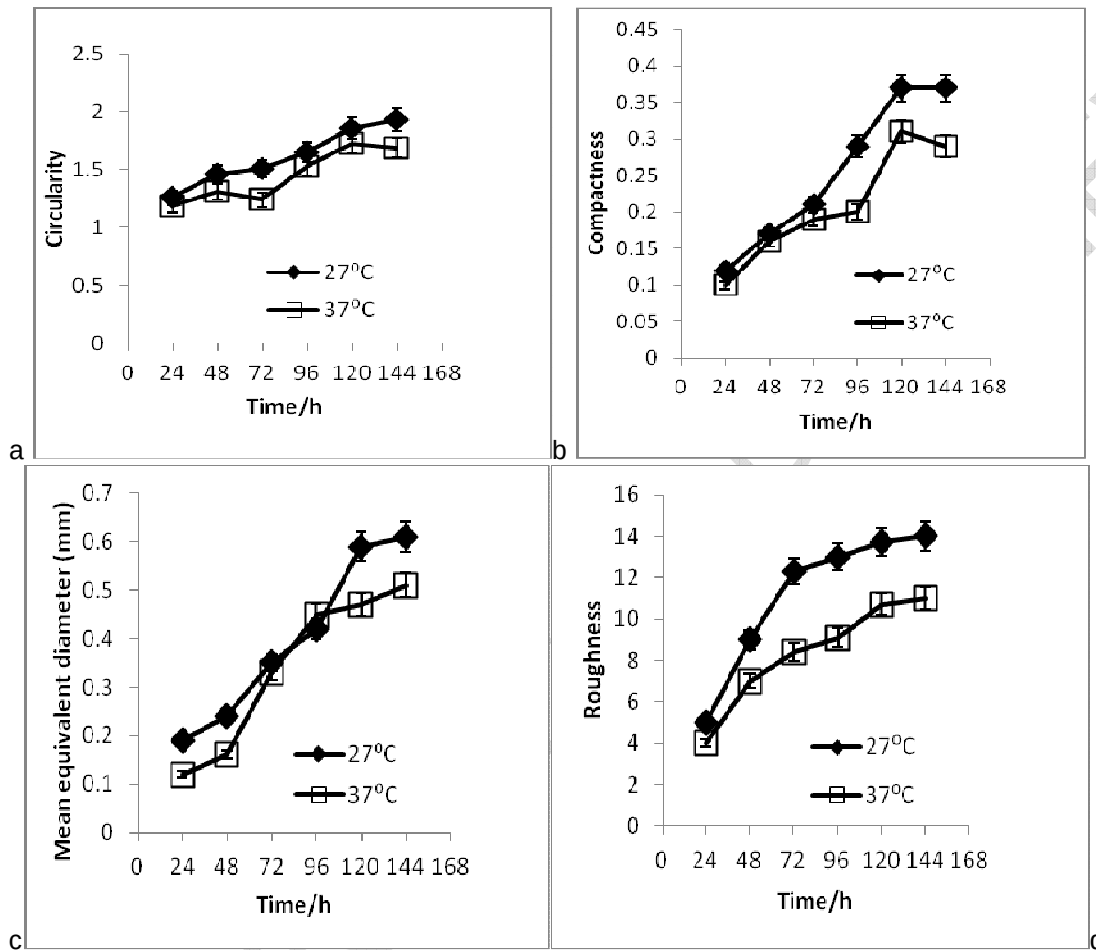


Fig.5 circularity (a), compactness (b), mean equivalent diameter (c), roughness (d) of *Aspergillus carbonarius* in shake flask cultures at different temperatures (27°C. And 37°C)

3.3 Thermal influence on culture variables

Figure 6 (a,b,c,d) shows the time profile of RSDA activity, total protein, biomass concentration and pH at varying temperature conditions. At 27°C, RSDA activity increased steadily reaching a peak of 293U/ml at 96h of 27°C and then decreased to 75U/ml by the end of fermentation. At 37°C a maximum yield of 291U/ml at 72h of fermentation was obtained and decreased to 87U/ml at the end of fermentation. This is to say that increase in temperature did not influence amylase activity. It is possible that the high RSDA activity maintained at 37°C resulted from sheared off hair that came together (agglomerated) hence maintaining the pellet morphology form which is most appropriate for the enzyme formation. The pH dropped from 5.12 to 3.0 at 27°C by the 72h of fermentation and remained relatively constant while at 37°C, it dropped further to 2.5. Increased biomass and protein were obtained at high temperature and same was the effect on total protein. Fragmentation of hyphal elements and pellets during submerged fermentations often results in growth renewal since fragments may act as centres for new growth, enabling reseeding of the pellet populations (Papagianni 2004). This may account for the high biomass concentration at 37°C as well as increased enzyme activity.

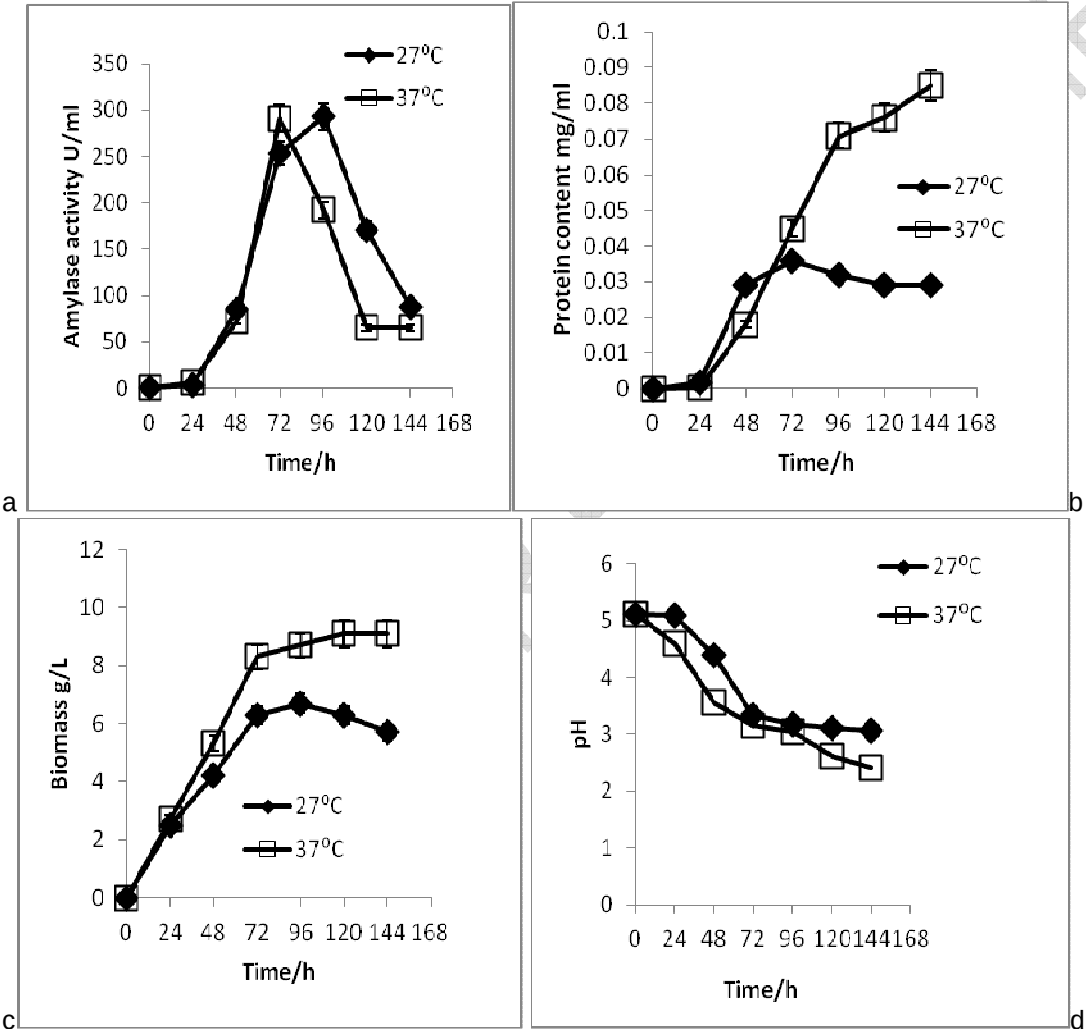


Fig. 6 Time course of RSDA activity (a), Protein content (b), and Biomass concentration (c) pH (d) in shake flask cultures of *Aspergillus carbonarius* at different temperatures 27°C and 37°C

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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