Research Paper

Effect of stress on growth, pigment production and morphology of *Monascus* sp. in solid cultures

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The aim of the current work was to study the influence of stress on pigment production in *Monascus purpureus* under solid-state fermentation. Thermal stress was induced by incubating the culture at various high temperatures. For giving osmotic stress, different concentration of NaCl and glycerol were added to the solid substrate prior to autoclaving. Morphological studies were done by light microscopy and scanning electron microscopy. When the cells were exposed to high temperature, high glycerol and salt concentration, significant changes in pigment production and growth were observed. High temperature (>45 °C) induced the production of more yellow pigments. High concentration of NaCl induced conidiation and caused a decrease in fungal biomass (up to 50%) but red pigment production increased from 11.86 OD/gds to 20.14 OD/gds. When subjected to glycerol stress, a significant increase in aerial mycelia was observed when compared with the control conditions. The results attain significance for exploiting the fungal culture of *Monascus purpureus* LPB 97 for producing colors of choice- red, or yellow, or increasing the yield of red pigments considerably. Thus, these results could well impact the commercial aspect of *Monascus* pigments for industrial application.

Keywords: *Monascus purpureus* / Pigment production / Growth / Industrial application

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Introduction

In nature, microorganisms are exposed to a constantly changing physical and chemical environment. However, microorganisms have evolved a variety of means by which they survive adverse stress conditions. It is widely accepted that biosynthesis of many secondary metabolites are induced by stress (Ranby and Rabek 1978). Under stress conditions primary metabolite conversion to secondary metabolites is a biochemical strategy through which the cell will be able to maintain energy sources and essential metabolites for a more favorable growth conditions. When an ordinary microorganism is exposed to stress such as elevation of culturing temperature, high osmotic pressure caused by a fermentative product, a high concentration medium ingredient or the like, or metabolic abnormality associated with the production of a target fermentative product, its growth may be inhibited or the productivity of the fermentative product may be reduced. But it has been proved that in production of useful substances such as amino acids by fermentation when cells are exposed to stress such as high temperature, high osmotic pressure, metabolic inhibition, presence of heavy metal, and viral infection, productivity of fermentative products in order to decrease the influence of stress which restrains growth of microorganisms and/or production of fermentative products so that the productivity and the yield are improved instead of being lowered (Kimura *et al.* 2002).

Several microorganisms have been described which accumulate large amounts of fermentative products when exposed to various stress conditions. Stresses can provide varying results because some bacteria may become more resistant or even more virulent under stresses through stress reactions such as synthesis of protective stress shock proteins (Leistner 2000). Some

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Dunaliella species were reported to accumulate large amounts of β-carotene when exposed to salt stress (Ben-Amotz and Avron 1983). Similar response has been reported in *Haematococcus pluvialis*, with the accumulation of red ketacarotenoid when exposed to higher light intensities and salt stress (Boussiba 2000, Cifuentes *et al.* 2003, Steinbrenner and Linden 2003). Similarly, Orosa *et al.* (2001) found that *Chlorella zofingiensis* produced increased amounts of asthaxanthin in response to a salt stress of 300 mM NaCl in comparison with non-stressed cells growing under high light irradiation. The biochemical potential of filamentous fungi and their adaptation to extreme life conditions in liquid fermentation have been exploited to produce molecules such as antibiotics (*e.g.*, penicillin, cephalosporins), enzymes (α-amylase, cellulase), organic acids (*e.g.*, citric acid) (Kubicek and Rohr 1986, Moreika *et al.* 1996) and food colorants (*e.g.*, Anka) (Lin 1973, Pandey *et al.* 2001). Yet little is known about the physiological and morphological response of filamentous fungi to various environmental stresses under solid-state fermentation.

It has long been known that micro-organisms of the genus *Monascus* produces at least six major related pigments which could be categorized as (1) orange pigments: rubropunctatin ($C_{21}H_{22}O_5$) and monascorubin ($C_{23}H_{26}O_5$); (2) yellow pigments: monascin ($C_{21}H_{26}O_5$) and ankaflavin ($C_{23}H_{30}O_5$); and (3) red pigments : rubropunctamine ($C_{21}H_{23}NO_4$) and monoscorubramine ($C_{23}H_{27}NO_4$) (Sweeny *et al*. 1981). *Monascus* pigments are a group of secondary fungal metabolites called azaphilones, which have similar molecular structures as well as similar chemical properties (Carvalho *et al*. 2003). No effort has been made so far to induce stressed fermentation conditions to enhance pigment production. The purpose of this study was to investigate the effect of stress conditions on growth and pigment production by *Monascus purpureus* under solid-state fermentation.

Materials and methods

Microorganism and growth conditions

A culture of *Monascus purpureus* LPB 97 was used in the present study. It was maintained on yeast extract – peptone – glucose medium (Hi-Media, Bombay), preserved at 4 °C and sub-cultured once in every three weeks.

Inoculum preparation

To fully sporulated (8-days-old) agar slope culture, 10 ml of sterile distilled water was added. The spores were scrapped under strict aseptic conditions. Filtration of this suspension through sterile glass wool resulted in suspension containing unclumped spores with no hyphae. This filtrate was used as the inoculum.

Solid-state fermentation

Experiments were conducted in 250 ml Erlenmeyer flasks containing 5 g jack fruit seed powder. The substrate was moistened with salt solution and distilled water in such a way as to obtain final moisture content of 50–60%. After thorough mixing, the wet substrates were autoclaved at 121 °C for 20 min and cooled to room temperature. It was inoculated with the spore suspension containing 1.5×10^5 spores per ml of *Monascus purpureus* LPB97 and incubated at 30 °C for 7 days. Unless and otherwise mentioned, these conditions were maintained throughout the experiment. Thermal stress experiments were carried out by incubating the flasks at various incubation temperatures (25, 30, 35 40 and 45 °C). In a separate experiment to investigate the effect of thermal stress on spores, spore suspension was subjected to different temperature (50, 60, 70 and 80 °C) before inoculation. For this, spore suspension was prepared as described above, diluted to 10^8 spores per ml and placed in a water bath (Julabo, USA) at set temperature. At the start of the experiment, the temperature of the spores suspension was monitored and when it reached the experimental temperature, was allowed to stand for 1 min. A 100 µl portion of each heat treated suspension was plated on YPG plates and was kept for incubation at 30 °C for 3 days. The effect of thermal stress on spore viability was evaluated by the plate count method for determining colony-forming units. Colonies were counted after they reached macroscopic size. The heat treated spore suspension was then used to inoculate the substrate and flasks were incubation at 30 °C. For the salt stress experiments, different concentration of NaCl – 6, 8, 10 and 12% (w/w) were added to the solid substrate prior to autoclaving and incubation period was extended up to 10 days. The influence of glycerol stress was investigated by adding glycerol at different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 M) to the solid substrate. Control experiments were conducted in parallel to the stress experiments for comparison.

Effect of stress on fungal morphology

To study the effect of stress on morphology, YPG plate cultures (stress conditions) were incubated for 10 days. After 10 days colony mycelium were observed directly under light microscope (Leica Microsystems GmbH, Germany) and photographed. The colonies were examined for morphological details with a scanning electron

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microscope (JEOL JSM 5600LV). SEM digitized photographs were obtained with a magnification 1000-times using an accelerator voltage of 10 kV.

Pigment extraction

From the fermented solid substrate, a known amount was taken for pigment extraction using 90% ethanol and mixed (5 ml of solvent per gram of fermented material) on a rotary shaker at 200 rpm for 1 h, allowed to stand for 15 min and filtered through Whatman #1 filter paper.

Pigment estimation

The analysis of pigment production was done by measuring absorbance maxima (λ_{max}) of pigment extract by spectral analysis (Lin and Demain 1992) using a double beam spectrophotometer (Shimadzu, UV 1601), taking in to consideration the dilution factor of the sample (Chiu and Poon 1993). Only extra-cellular pigments were considered in this study. Pigment yield was expressed as OD at its λ_{max} per g dry fermented matter (Johns and Stuart 1991).

Biomass estimation

The growth of fungal culture was estimated by determining the N-acetyl glucosamine released by the acid hydrolysis of the chitin, present in the cell wall of the fungi (Sakurai *et al.* 1977). Acid hydrolysis sample (1 ml) was mixed with 1ml acetyl acetone reagent and incubated in a boiling water bath for 20 min. After cooling, ethanol (6 ml) was added, followed by the addition of 1 ml of Ehrlich reagent and incubated at 65 °C for 10 min. After cooling, the optical density was read at 530 nm against the reagent blank. N-Acetyl glucosamine (Sigma) was used as the standard.

Results and discussion

Effect of thermal stress

For most of the micro-organisms, temperature is a critical environmental signal for regulating developmental and physiological process. Thermal stress studies were conducted in two ways, by incubating the fermenting matter at different temperatures, and by exposing (treating) spores at different temperatures (heat shock). Therefore, in the first set of experiments, fermentation was carried out at different incubation temperatures ranging from 25–45 °C in order to determine the effect of thermal stress on growth and pigment yield. From the spectral analysis (Fig. 1), it was found that maximum absorbance at 500 nm was obtained at 30 °C. But beyond 30 °C, the spectral lines shifted towards 400 nm, which corresponded to yellow pigments. Although biomass yield was very poor at 40 °C (15 mg glucosamine/gds) when compared to other incubation temperatures (data not shown), there was an increase in yellow pigment production (32 OD/gds), which was more than that produced at 30 °C (24 OD/gds). Beyond 40 °C, the yellow pigment production decreased drastically. This result was similar to the findings of Carvalho *et al.* (2005), who also reported a shift in absorbance maxima of pigment extract at different incubation temperature.

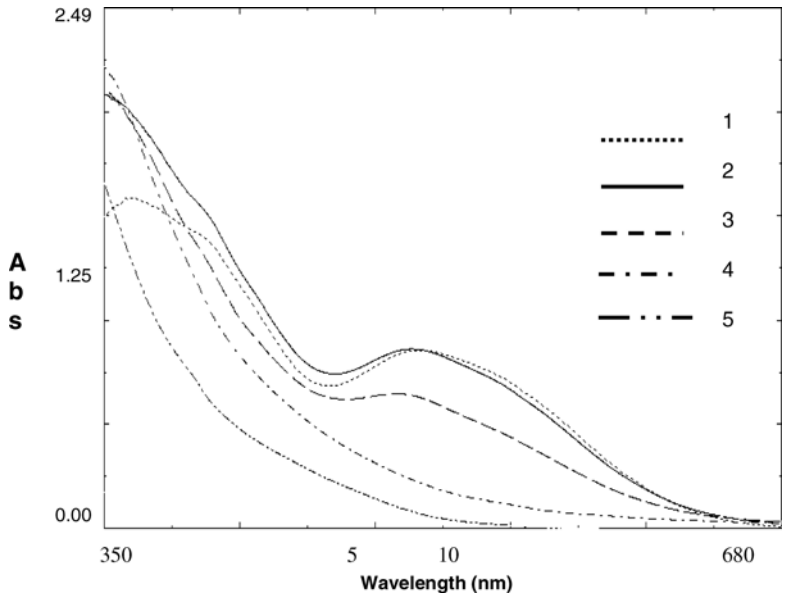

Figure 1. Spectrum of pigment extract at different incubation temperature. (1) 25 °C, (2) 30 °C, (3) 35 °C, (4) 40 °C, (5) 45 °C.

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In another set of experiments spores were given heat shock and subsequently fermentation was carried out. A sample was taken to check the viability of heat treated spores. Spores treated at 40 °C and 50 °C showed viability on plate after two days and the number of single colonies formed was almost equal to that determined for the untreated control (results not shown). Spores exposed to 60 °C and 70 °C showed delayed germination (~24 h delay in comparison to control) and those treated at 80 °C did not grow, showing complete loss of viability (results not shown). A similar observation was reported by Abrashav *et al.* (2005), with conidiospores of *Aspergillus niger*. Figure 2 shows the growth and pigment formation by the *M. purpureus* spores exposed to high temperature (heat shock). Interestingly, although biomass production increased when the spores were treated at 50 °C, pigment production remained comparable to the control experiment. However, at higher temperatures, growth was reduced drastically with no growth at 80 °C. Conversely, pigment yield showed a reduction at 60 °C, but increased remarkably under a heat shock at 70 °C. It has been reported that following exposure to elevated temperatures, many organisms rapidly synthesize a highly conserved set of proteins termed heat shock proteins and their induction appears to correlate with the adaptation of the organism to hypothermic stress condition (Schlesinger 1990). Further studies have to be carried out to find out any relationship between enhanced pigment production on giving heat shock to fungal spores and involvement of any heat shock proteins in that process.

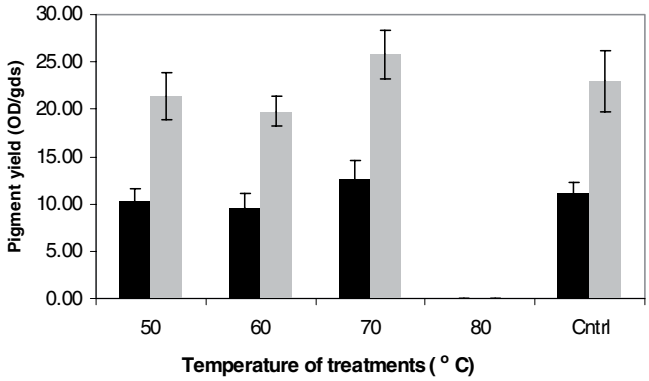

Figure 2. Effect of giving heat shock to spores on pigment yield; (■) yield of red pigment, with λ_{max} at 510 nm, (□) yield of yellow pigments, with with λ_{max} at 406 nm. Values are mean of duplicate measures from two fermentations ± SD.

Effect of salt stress on growth and pigment production

When the culture was exposed to different concentrations of salt stressed fermentation condition, the spectral analysis of the pigment extract showed a shift in absorbance maxima from the orange region (410 nm) towards the red region (430 nm), showing a tendency to produce more red pigments at high salt concentration (Fig. 3). The pigment yield and biomass obtained from *M. purpureus* LPB97 for different concentration of NaCl in SSF medium grown for a period of 12 days were shown in Fig. 4. When compared to control, there was a slight increase in pigment yield and biomass at 6% and 10% (w/w) NaCl. This observation suggested that low concentrations of salt appeared to promote the growth of the fungal culture. With further increase in salt concentration, however, there was a drastic reduction in biomass production. At 10% NaCl concentration, red pigment production increased from 11.86 OD/gds to 20.14 OD/gds. The pigment with its absorbance maxima at 430 nm was also increased at this high salt stress condition. Similar stimulating effect of NaCl was reported by Vigants *et al.* (1995). Present results were in

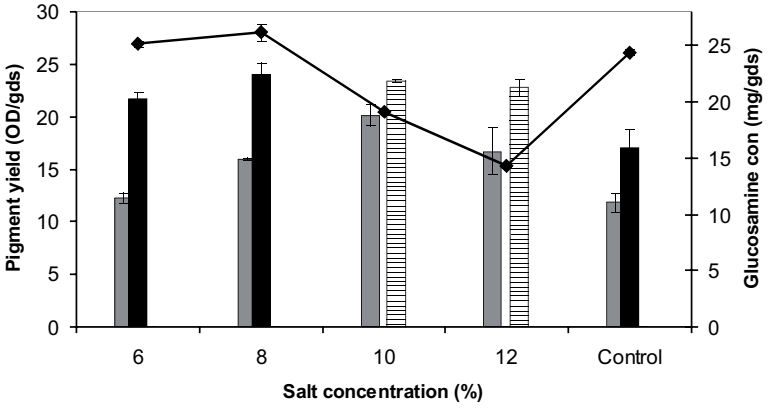

Figure 3. Effect of inducing salt stress on growth and pigment yield. (■) Yield of red pigment with λ_{max} at 510 nm, (■) yield of orange pigment with λ_{max} at 410 nm, (▤) yield of orange-red pigment with λ_{max} at 430 nm, (—◆—) biomass at different NaCl concentration. Values are mean of duplicate measures from two fermentations ± SD.

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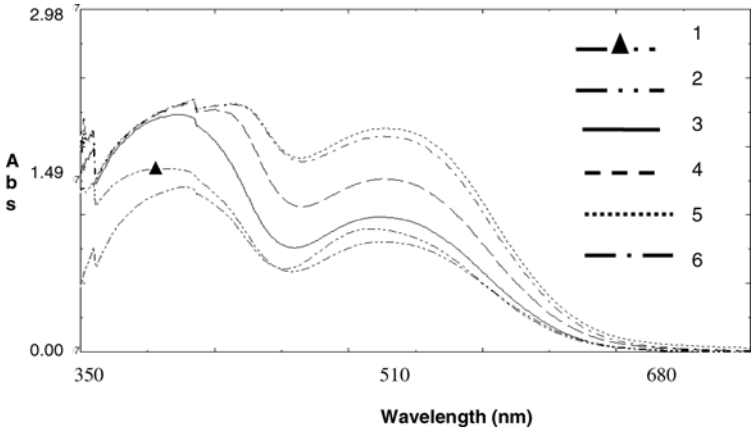

Figure 4. Spectrum of pigment extracts showing the effect of NaCl concentration (1) Control, (2) 4% NaCl (w/w), (3) 6% NaCl (w/w), (4) 8% NaCl (w/w), (5) 10% NaCl (w/w), (6) 12% NaCl (w/w).

agreement with Khaware *et al.* (1996) who reported the response of *Candida membranofaciens* to NaCl stress by increased production of glutamic and aspartic amino acids. The increased production of pigments in the presence of NaCl could be due to an osmoprotective role of pigments in the organism. As salt reduces water activity considerably, the response of the fungi towards salt stress is governed by the demands imposed on the water activity by the organism (Ingram and Kitchell 1967).

The results obtained for salt stress versus incubation time are shown in Fig. 5. When compared to control, under salt stress condition, there was a significant delay in growth and pigment production. There was a lag phase in growth 72 h and pigment production started only at 120 h, whereas in control, growth was evident at 24 h and pigment production at 48 h onwards. The generalized nature of inhibition pattern in growth under salt stress was in agreement with the results of Alder *et al*. (1985), Larsson and Gusfafsson (1993), Neves *et al*. (1997), Obuekwe *et al.* (2005). It is known that microorganisms under stress are less efficient in the conversion of substrates into microbial biomass synthesis (Bardgett and Saggar 1994). Salt stress can imbalance the osmotic potential in fungi cells generating a water deficit and the influx of sodium may lead to metabolic toxicity which could be the reason for the drastic reduction in biomass.

Effect of glycerol stress on growth and pigment production

The pigment yield and biomass obtained by *M. purpureus* LPB97 for different concentration of Glycerol are shown in Fig. 6. Although glycerol could induce osmotic stress in the microorganisms, the response in the present study was different from one that produced by NaCl. Results shown in Fig. 6 clearly showed the gradual increase in both pigment yield and biomass with the increase in glycerol concentration. This increase in growth seemed contradictory to the general observation

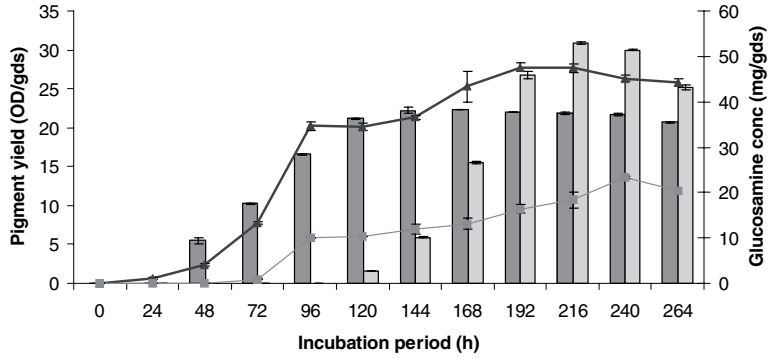

Figure 5. Time course of growth and pigment yield under salt stress *vs.* control (▥) yield of red pigment with λ_{max} at 510 nm produced under control condition, (▩) yield of red pigment with λ_{max} 510 nm produced under salt stressed condition, (–▲–) biomass under control condition, (–●–) biomass under salt stressed condition. Values are mean of duplicate measures from two fermentations ± SD.

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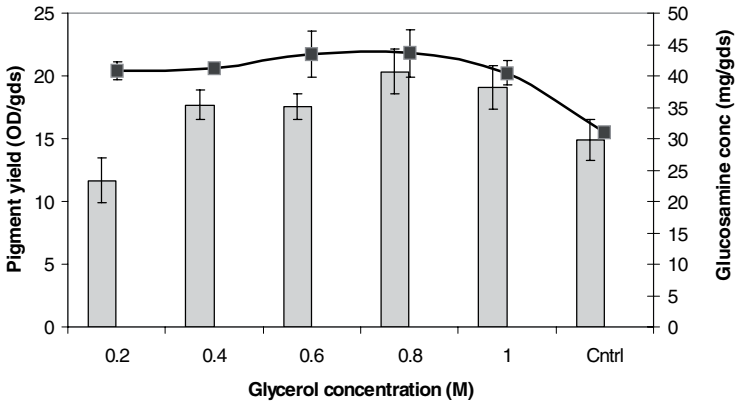

Figure 6. Effect of inducing glycerol stress on growth and pigment yield (▨) of red pigment with λ_{max} at 510 nm (–■–) biomass. Values are mean of duplicate measures from two fermentations ± SD.

tion that microorganisms under stress would be less efficient in the conversion of substrates into microbial biomass. Apparently, the positive effect could be due to the possibility that glycerol not only acted as an osmolyte, it also served as a carbon source and had a metabolic function (Ansell *et al*. 1997).

Effect of stress on fungal morphology

Colony formation was observed on YPG plates under different stress conditions (Fig. 7-1A, 1B, 1C, 1D). Evidently the size of colonies reduced considerably under temperature stress (43 °C) and the color of the colony was also different yellow (1B) (normal colony color being red). Microscopic examination (4×) revealed the growing front of the colony as a tuft of yellow mass. Further examination (40×) revealed thick walled pigmented mycelium and reproductive hyphae with accumulation of dark yellow pigments (Fig. 7-3A). Thickening of the cell wall could transiently protect the cells by absorbing thermal energy (Rosas and Casadevall

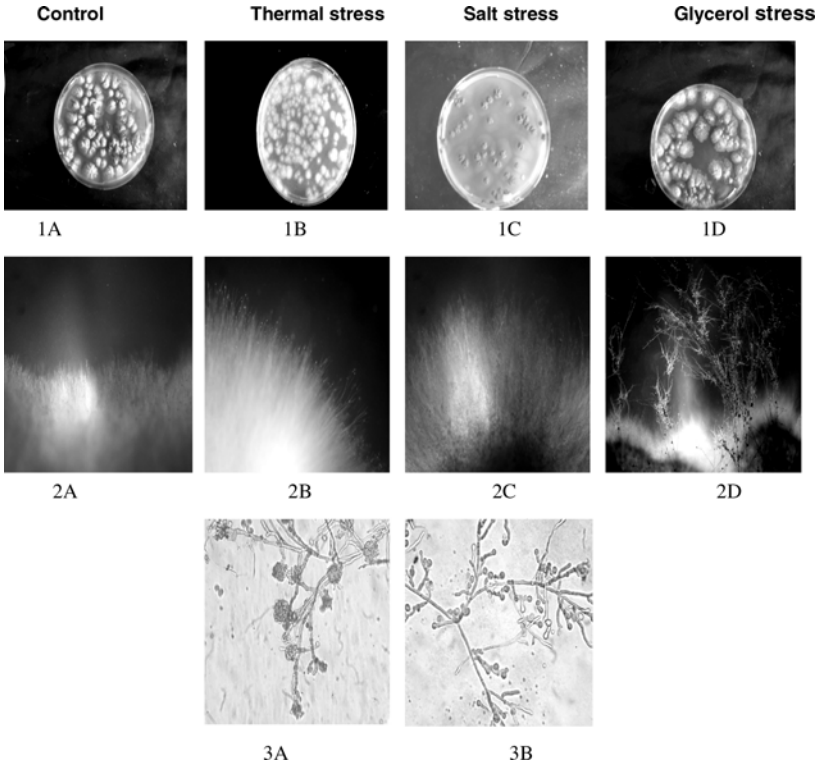

Figure 7. Effect of stress on morphology of 10-day-old *Monascus* culture, (1) Colony grown in YPG agar (2) light microscopic observation of growing front of the colony (4×), (3) light microscopic observation of mycelium (40×), (A) control, (B) thermal stress, (C) salt stress, (D) glycerol stress.

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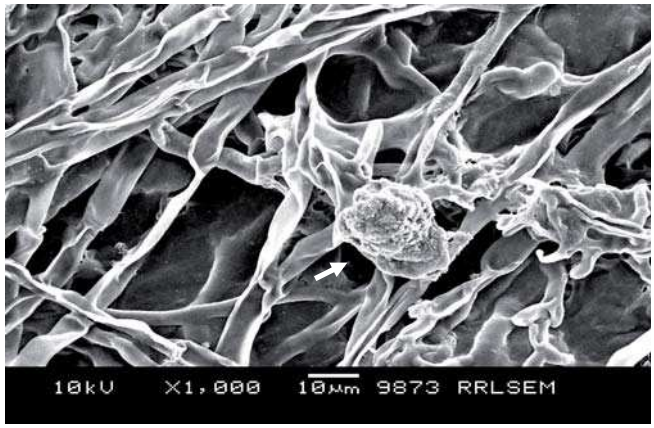
1997, Han *et al*. 2004). *M. purpureus* is a mesophilic fungus with optimum growth temperature below 35 °C and this morphological change could be a consequence of thermal stress to the culture. Salt stress resulted in reduction in colony size showing strong inhibition of hyphal extension and accumulation of red pigments (Fig. 7-1C). The growing front of the colony (4×) showed thick walled filaments embedded with numerous spores (Fig. 7-2C). *Monascus*, a homothallic fungus, is able to produce spores asexually as well as sexually. Under normal condition, *M. purpureus* forms oval, or pyriform aleiuroconidia, single, or in a chain up to 3–4. But under salt stress, in 10-day-old culture showed cluster of thick walled conidia with pigment accumulation (Fig. 7-3B). It has been reported that conidiospores have the capacity to survive sudden, often drastic and stressful changes in their environment (Shinohara *et al*. 2002). Further confirmation of salt induced change in fungal morphology was achieved by scanning electron microscopy (Fig. 8B) SEM elucidated the clustered spores throughout the length of the hyphae, clearly showing that it was mycelium itself that had transferred in to these thick walled structures. Spore formation has been generally accepted as a response to environmentally adverse conditions (Suh and Shin 2000). It has been reported that when the walls of *Monascus* cells suffer from hydrolysis, *Monascus* cells overproduce hydrophobic substances such as pigments for blocking these enzymatic attacks (Shin *et al*. 1998). In the present study, since the organism was subjected to extreme saline stress, thick walled mycelium and overproduction of hydrophobic pigments could be a possible way to encounter the loss of water from the cytoplasm to the surrounding medium. It is difficult to determine if reproduction of *Monascus* cells followed completion of asexual cycle and initiation of sexual cycle because asci were observed even in the early cultivation period. But under salt stress, the development of asci was observed very late in the cultivation period. Colony was relatively spread under glycerol stress (Fig. 7-1D). Microscopic observation of growing front of the colony (4x) showed a significant increase in aerial mycelia under glycerol stress (Fig. 7-2D) when compared with the control (Fig. 7-2A). Hyper-branched hyphal tips under glycerol stress may be the result of the inability of the organism to maintain positive turgor pressure (Bartnicki-Garcia *et al.* 2000), thus inducing premature branching. Microscopically the mycelium showed both sexual and asexual spores under glycerol stress. The understanding of cell morphology is a key to enhancing and optimizing product activity (O'Shea and Walsh 1996).



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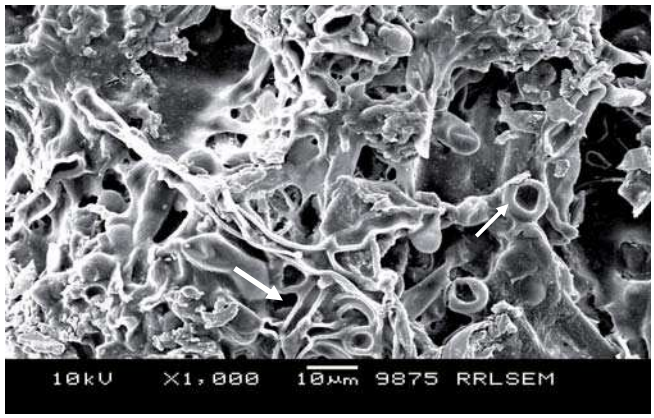

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Figure 8. Scanning Electron Microscopic (SEM) picture showing thick-walled conidial clusters. (A) Control, arrow show single conidium, (B) arrows showing cluster of thick walled conidia under salt stress.

The most significant outcome of this study was an increased yield of pigments from *M. purpureus* LPB97 by inducing stress conditions. It could be shown that *Monascus* responded to thermal and osmotic stress by producing more pigments, thereby showing the thermoprotective and osmoprotective role of pigments in the organism. Significant change in fungal morphology was also observed in response to stress. Therefore, it could be possible to relate the stressed environmental conditions to growth and product optimization. Thus, it could be concluded that *Monascus* species were sensitive to detect stress environmental conditions and possessed mechanism to survive those conditions. The results attain significance for exploiting the fungal culture of *Monascus purpureus* LPB 97 for producing colors of choice – red, or yellow, or increasing the yield of red

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pigments considerably. Thus, these results could well impact the commercial aspect of *Monascus* pigments for industrial application.

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