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Solid-state fermentation for the production of *Monascus* pigments from jackfruit seed

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Abstract

The aim of the present work was to investigate the feasibility of jackfruit seed powder as a substrate for the production of pigments by *Monascus purpureus* in solid-state fermentation (SSF). A pigment yield of 25 OD Units/g dry fermented substrate was achieved by employing jackfruit seed powder with optimized process parameters such as 50% initial moisture content, incubation temperature 30 °C, 9×10^4 spores/g dry substrate inoculum and an incubation period of seven days. The color of the pigments was stable over a wide range of pH, apparently due to the buffering nature of the substrate, which could be a significant point for its scope in food applications. To the best of our knowledge this is the first report on pigment production using jackfruit seed powder in solid-state fermentation (SSF).

Keywords: Solid-state fermentation; *Monascus purpureus*; Pigments; Jackfruit seed powder

1. Introduction

Color and flavor are the signals that are immediately perceived by the optical and chemical senses of humans and these attributes determine whether a certain food is appealing. Attractive food colors and flavors are usually translated into increased consumption, which is a fundamental behavioral response. However, color and flavors are often sensitive to heat, oxygen, light and acid and thus changed or lost during processing and storage. Natural colorants and flavors mainly derived from plants and chemosynthetic compounds are used by the food industry to replenish and sometimes raise the genuine stock (Pandey et al., 2001). Recent increasing concern on the use of edible coloring agents has banned various synthetic coloring agents, which have a potential of carcinogenicity and/or teratogenicity (Fabre et al., 1993). This circumstance has inevitably increased demands for highly safe, naturally occurring edible coloring agents, one of which is *Monascus*-pigment (Francis, 1987).

It has long been known that microorganisms of the genus *Monascus* produce red pigments, which can be used for coloring foods. In certain oriental countries, microorganisms of this type are grown on grains of rice and once the grains of rice are penetrated by the red mycelium, the whole matter is finely ground. Although for years it has been known that there are six pigments, in the last decade some new pigments have been discovered, which included xanthomonascin and yellow II, possibly derived from rubropunctatin (Sato, 1992; Juzlova et al., 1996; Watanabe et al., 1997). According to some authors, there are more than ten pigments, although only some of them have the structure elucidated (Shin et al., 1998). The orange pigments,

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monascorubrin and rubropunctatin, are synthesized on the cytosol from acetyl coenzyme A, through the multienzymatic complex polyketide synthase. These pigments have a structure responsible by their high affinity to compounds containing primary amino groups (thus called aminophiles). Reactions with amino acids lead to formations of hydro soluble red pigments, monascorubramine and rubropunctamine. The mechanism of yellow pigment formations is not yet clear; some authors consider that these are product of the alteration of orange pigments, as others believe it to be pigments with their own metabolic pathway (Carvalho et al., 2003).

In this regard, recent research has been devoted to the studies on general culture conditions and substrates evaluation for pigments production, and among these, most of the studies have been performed at laboratory-scale using liquid culture medium, which presents easily controllable conditions. Due to high cost of currently used technology of pigment production on an industrial-scale, there is a need for developing low cost process for the production of pigments, which could replace synthetic pigments. From the literature, it is evident that utilization of a cheaply available substrate through solid-state fermentation (SSF) could attain such an objective. In SSF process, the solid substrates not only supplies the nutrients to the microbial culture growing in it, but also serves as an anchorage for the cells (Pandey, 2003). In recent years, SSF has gained much interest for the production of primary and secondary metabolites. SSF presents a more adequate habitat for fungus, with high pigment productivity in a relatively low-cost process by using agro-industrial residues as substrates (Pandey et al., 2000, 2001).

Various agro-industrial residues such as rice bran, wheat bran, cassava, etc. have been used for pigment production. However, no effort has been made so far to utilize jackfruit seed as a substrate for pigment production. Largest of all tree-borne fruits, the jackfruit could be 20–90 cm long and 15–50 cm wide, and the weight could be from 4.5 to 20 kg, or even as much as 50 kg (Morton, 1987). Jackfruit is popular in several tropical countries and is an excellent example of a food prized in some areas of the world (mostly tropical) and is available in large part of the year at the places where produced. However, in some locations is the world, it is not used as food material and allowed to go to waste. There may be 100, or up to 300 seeds in a single fruit. Seeds make up around 10–15% of the total fruit weight and have high carbohydrate and protein contents (Bobbio et al., 1978; Kumar et al., 1988). Jackfruit seeds are discarded as waste from different agro-industries and higher percentage of fruits. Seeds also go waste from the fallen fruits. Since the seeds are rich in carbohydrate, protein and trace elements, it can be used as a potential substrate for the production of food grade red pigments.

Thus, the main objective of this study was to develop a potential fermentation process for the production of pigments employing SSF using non-conventional agro-residues and also studying the effect of different parameters in an attempt to maximize pigment production.

2. Methods

2.1. Culture

A culture of *Monascus purpureus* LPB 97, available in the Process Biotechnology Laboratory, Federal University of Parana, Brazil, was used in the present study. It was maintained on yeast extract–peptone–glucose medium (Hi-Media, Mumbai, India); preserved at 4 °C and sub-cultured once in every three weeks.

2.2. Inoculum preparation

To fully sporulated (6–8 days old) agar slope culture, 10 ml of sterile distilled water was added. Then the spores were scrapped under aseptic conditions. The spore suspension obtained was used as the inoculum (1.5×10^5 spores/ml).

2.3. Substrate and solid-state fermentation

Experiments were conducted in 250 ml Erlenmeyer flasks containing 5 g substrate (powdered jackfruit seed). The substrate was moistened with salt solution and distilled water in such a way as to obtain final moisture content of 60% After thorough mixing, the wet substrates were autoclaved at 121 °C for 20 min and cooled to room temperature. It was inoculated with 2 ml the spore suspension containing 1.5×10^5 spores/ml of *M. purpureus* LPB97 and incubated at 30 °C for 7 days. Unless and otherwise mentioned, these conditions were maintained throughout the experiment.

2.4. Pigment extraction

From the fermented solid substrate, a known amount was taken for pigment extraction using 90% methanol (5 mL of solvent per gram of wet fermented material). The mixture was kept on a rotary shaker at 200 rpm for one hour, allowed to stand for 15 min and filtered through Whatman #1 filter paper.

2.5. Pigment estimation

Pigment estimation was done as described by Tseng et al. (2000) in which the optical density at its absorbance maxima were expressed as the concentration of pigment produced. 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 gram dry fermented matter (Johns and Stuart, 1991).

2.6. 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 1 ml 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.

2.7. Analytical methods

Reducing sugars were estimated by the dinitrosalicylic acid (DNS) method of Miller (1959). Enzyme profile at different incubation time was also monitored. α-Amylase activity was determined by the method of Okolo et al., 1995). The reaction mixture consisted of 1.25 ml of 1% soluble starch, 0.25 ml of 0.1 M acetate buffer (pH 5.0), 0.25 ml of distilled water, and 0.25 ml of crude water soluble extract. After 10 min of incubation at 50 °C, the liberated reducing sugars (glucose equivalents) were estimated by the method of Miller (1959). The color developed was read at 575 nm using a Shimadzu UV-160 A spectrophotometer. Glucose was used as the standard. The blank contained 0.5 ml of 0.1 M acetate buffer (pH 5.0), 1.25 ml of 1% starch solution and 0.25 ml of distilled water. One unit (IU) of α-amylase was defined as the amount of enzyme releasing 1 μmol glucose equivalent per minute under the assay conditions. Glucoamylase activity was determined by incubating a mixture of 0.5 ml aliquot each of extract and 1% soluble starch dissolved in 0.1 M sodium acetate buffer, pH 5 at 55 °C for 15 min (Bernfield, 1955). The reducing sugar released after 10 min was measured by the method of Miller (1959). One unit (U) of glucoamylase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar as glucose, per minute, under assay conditions and expressed as U/g of dry substrate (gds). Incubation time for maximum pigment production was estimated by spectral analysis of pigment extract at different incubation time.

2.8. Screening of agro-industrial residues

Tropical agro-industrial residues such as rice bran, wheat bran, coconut oil cake, sesame oil cake, palm kernel cake, groundnut oil cake, cassava powder, spent brewing grain, jackfruit seed powder, and tamarind seed powder were screened for selecting the best substrate for pigment production. These substrates were taken at a known quantity (5 g, mixed particle size, dry wt basis). The fermentation was conducted as described above.

2.9. Effect of incubation time, initial moisture, incubation temperature, inoculum size and initial pH of the substrate on pigment production

To study the effect of incubation period on pigment production, flasks were incubated for varying periods (24, 48, 72, 96, 120, 144, 168 and 192 h). Growth kinetics was studied by estimating the biomass and reducing sugar at various incubation periods and by relating the pigment producing capability of the fungus. Different initial moisture levels (40–60%) were employed in the substrate by adjusting the volume of distilled water to study their effect on pigment production. The fermentation was conducted as described earlier. Activity of α-amylase and glucoamylase were also monitored in the fermented samples with different initial moisture levels, as per the methods described earlier. To study the effect of incubation temperature on the growth of the culture and pigment production, the flasks were incubated at various temperatures (25, 30, 35, 40 and 45 °C). The effect of inoculum on pigment production was studied by adding different concentrations of inoculum size to the substrate.

pH of the substrate was changed by adjusting the pH of the salt solution and was optimized so as to get the required pH after autoclaving. The effect of initial pH of the substrate on pigment production was estimated by spectral analysis of the pigment extracts.

3. Results and discussion

3.1. Selection of substrate

Tropical agro-industrial residues such as rice bran, wheat bran, coconut oil cake, sesame oil cake, palm kernel cake, groundnut oil cake, cassava powder, spent brewing grain, jackfruit seed powder and tamarind seed powder were evaluated for selecting the best substrate for pigment production. Highest pigment production was achieved with jackfruit seed powder (12.113 OD Units/gds). Others either yielded negligible (sesame oil cake and coconut oil cake) or showed poor yields. (wheat bran 3.525, groundnut oil cake 0.150, sesame oil cake 0.375, palm kernel cake 7.650, coconut oil cake 0.118, spent brewing grain 4.356, tamarind seed powder 1.146, cassava flour 1.458, wheat bran 3.525 OD Units/gds). Hence, jackfruit seed powder was selected and used for subsequent studies.

3.2. Effect of incubation time on pigment production

Fig. 1 showed that maximum pigment production was obtained after 144 h of incubation (10.2 OD/gds), after which there was a decrease in pigment production. Fig. 2 shows the growth kinetics as estimated by the biomass at different incubation period. From the results it was evident that a lag phase occurred up to 24 h; there was relatively less utilization of reducing sugar up to 48 h. When the

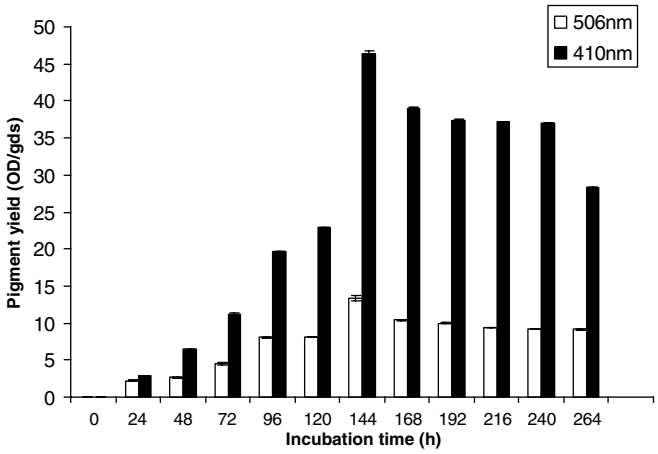

Fig. 1. Pigment production at different incubation period by *M. purpureus* LPB97.

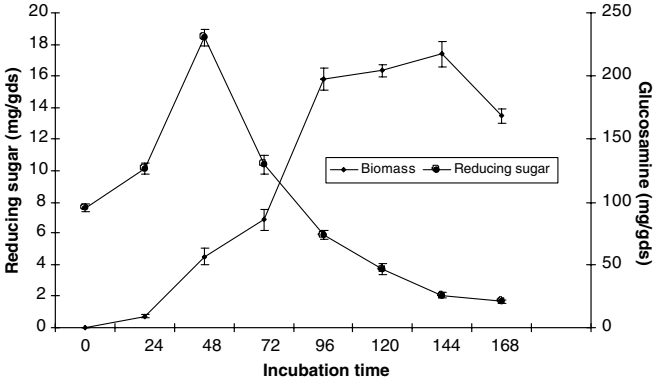

Fig. 2. Fermentation profile at different incubation period.

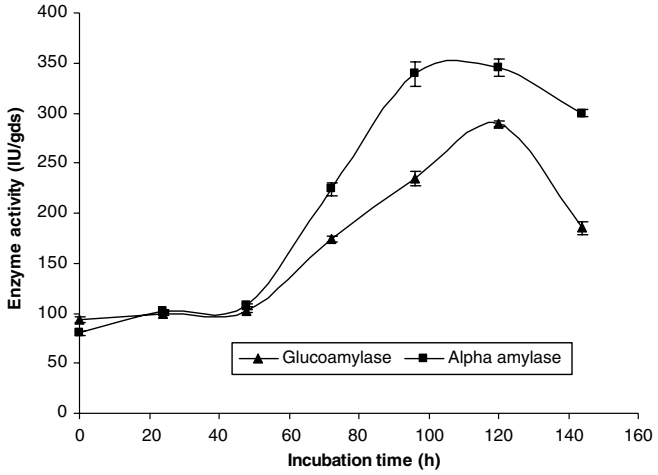

Fig. 3. Enzyme activities at different incubation period.

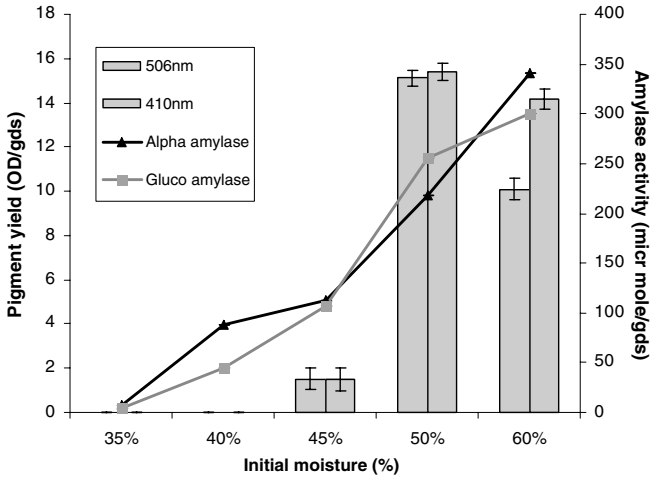

Fig. 4. Effect of initial moisture on pigment production by *M. purpureus* LPB97.

glucosamine synthesis started to increase from 48 h, the amount of soluble reducing sugar started to decrease correspondingly. Similarly, the incubation period from 144 to 168 h showed decreased fungal growth; the total soluble reducing sugars were totally exhausted by that time. The presence of two hydrolyzing enzymes, α-amylase and glucoamylase (Fig. 3) could be the reason for the large increase in reducing sugars up to 48 h.

3.3. Effect of initial moisture content on pigment production

For solid state fermentation, moisture is a key parameter to control the growth of microorganism and metabolite production (Pandey, 1992, 2003). The effect of initial moisture content of the substrate is presented in Fig. 4. Maximum pigment production was observed at 50% initial moisture content (15.12 OD Units in 144 h). A decrease in pigment yield was observed when the moisture level was higher, or lower than the optimum. This result was similar to the findings of Johns and Stuart (1991) who reported that initial substrate moisture content less than 40% gave less pigmentation, but that of 50–56% could give the highest pigmentation. Higher initial moisture in SSF leads to suboptimal product formation due to reduced mass transfer process and decrease in initial moisture level results in reduced solubility minimizes heat exchange, oxygen transfer and low availability of nutrients to the culture (Carrizales and Rodriguez, 1981). It has been reported that each *Monascus* strain has its own individual optimum initial moisture content for pigment production. Lotong and Suwanarit (1990) reported that at a high initial moisture content, *Monascus* sp. NP1 could liberate high glucose that can inhibit pigment production. Previous studies have shown that initial moisture content in the solid substrate is one of the key factor affecting glucoamylase activity as well as pigmentation of the *Monascus* (Yongsmith et al., 2000). In present study at an initial moisture content of 35 and 40%, *Monascus* showed low alpha amylase and glucoamylase activity. Growth was also poor at this moisture level (data not shown). At 50% substrate moisture content, higher alpha amylase and glucoamylase activity were observed. Activity of these hydrolyzing enzymes resulted in effective utilization of starchy substrate, which could be attributed to the high pigment yield at 50% initial moisture content. With further increase in initial moisture

alpha amylase and glucoamylase activity also increased. An increase in glucoamylase activity at high initial moisture up to 80% has been reported by Ellaiah et al. (2002) and increased alpha amylase activity at 70% initial moisture has been reported by Ramachandran et al. (2004). Reducing sugars rapidly liberated due to increased enzyme activity at this initial moisture level would have inhibited pigment production. In SSF, the intensity of microbial growth generally depends on the initial moisture level and it indirectly affects the production titre. In general, fungal cultures require low moisture level (20–70%) compared to the requirement of a bacterial culture, which is higher than 70%. Higher moisture content resulted in agglomeration of the substrate; subsequently restricting the supply of oxygen for microorganism for growth can also be a possible explanation for reduction in pigment production at high initial moisture (Gautam et al., 2002).

3.4. Effect of temperature on pigment production

Temperature is an important factor as it influences metabolic activities and microbial growth. From the results it was evident that maximum pigment production was obtained at 30 °C, which clearly indicated the mesophilic nature of the fungus (data not shown). This observation was in agreement with Domsch et al. (1980) who reported an optimum temperature between 30 and 37 °C for different isolates of *Monascus* sp. An interesting observation was made from the Fig. 5, that the maximum absorbance obtained at 500 nm at 30 °C shifted to 400 nm (which corresponded to yellow pigments), when incubation temperatures was higher than 30 °C. At 40 °C, there was maximum production of yellow pigments, which was more than those produced at 30 °C. Beyond 40 °C, the yellow pigment production also decreased drastically. These results were similar to the findings of Carvalho et al. (2005), who also reported a shift in absorbance maxima of pigments at different incubation temperature.

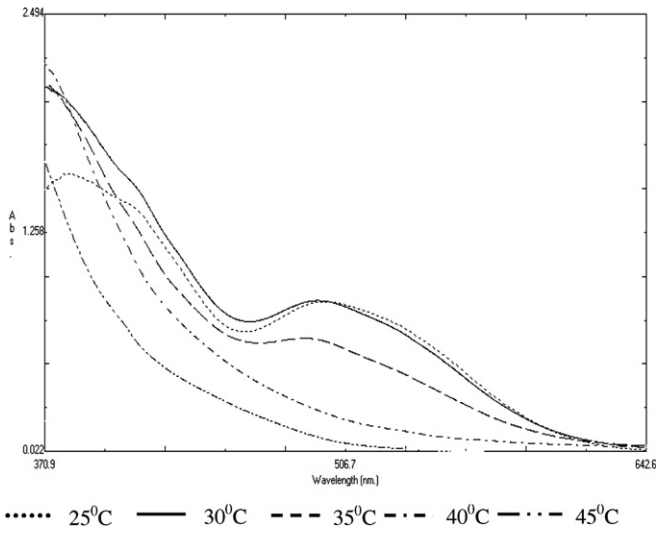

Fig. 5. Spectral analysis of pigment extract at different incubation temperature.

3.5. Effect of inoculum concentration on pigment production

Pigment production significantly increased to 25.45 OD Units/gds for an inoculum size of 3 ml spore suspension (9×10^4 spores/gram dry substrate). Results were in agreement with Pandey et al. (2000), which showed that at lower and higher inoculum levels, poor pigment production was observed. A low inoculum density resulted in insufficient biomass causing reduced product formation, whereas a higher inoculum produced too much biomass and depleted the substrate of nutrients necessary for product formation (Fig. 6).

3.6. Effect of initial pH of the medium on pigment production

It was observed that the pH of the medium changed significantly after autoclaving (data not shown). pH of the salt solution was optimized so as to get the desired pH after autoclaving. Thus, in order to achieve a pH of 2 after autoclaving pH of the salt was reduced to 0.3. Similarly to get a pH of 7.5, the salt solution was adjusted to high alkaline pH of 12.9 (data not shown). From the spectral analysis (Fig. 7) it was observed that for pH 3, the absorbance maximum was obtained at 469 nm. For pH 3.5 and 4, the λ max shifted towards left giving the peak values at 445 and 400 nm, respectively. It has been reported that at lower pH, there is predominance of yellow pigments and at a higher pH, there is a predominance of red pigments (Yongsmith et al., 1993). But in present study, over a wide range of pH, i.e. from 4.5 to 7.5, though pigment yield differed, they all yielded pigments with similar absorption peaks around 410 nm and 510 nm. At very low pH of 2 and 2.5, there was no fungal growth; the maximum growth was attained at pH 4. Thereafter there was decrease in growth profile even though pigment yield gradually increased from pH 4.5 to 7.5 (Fig. 8). These results showed that the color of the pigment

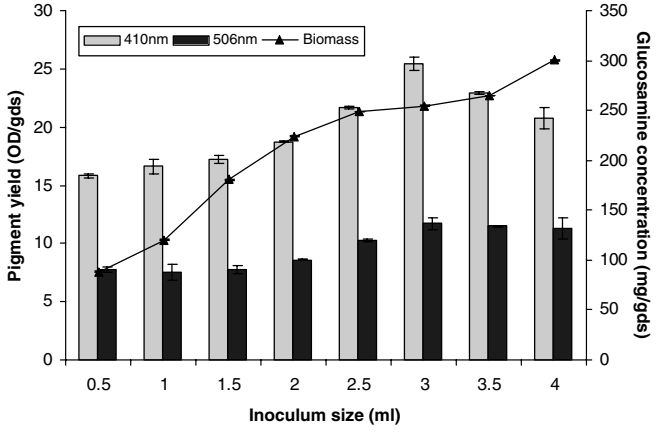

Fig. 6. Effect of inoculum size on growth and pigment production by *M. purpureus* LPB97 under SSF.

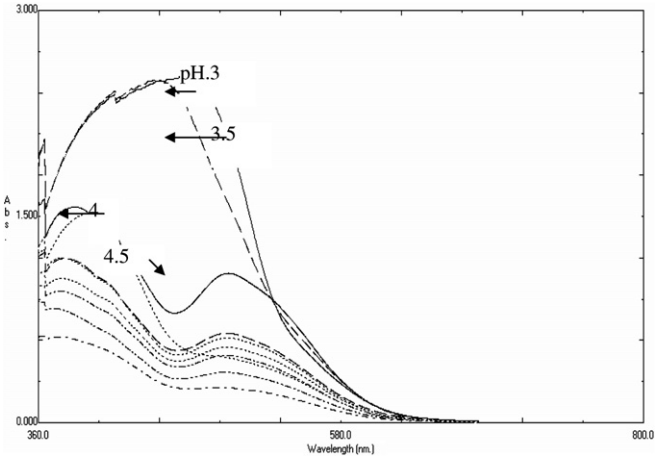

Fig. 7. Spectral analysis of pigment extracts of different initial pH of the substrate.

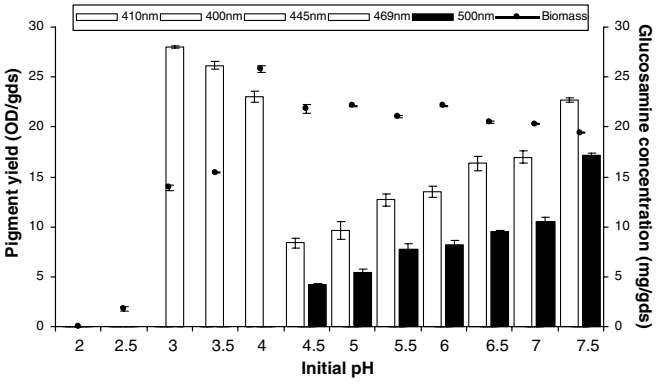

Fig. 8. Effect of initial pH of substrate on growth and pigment yield.

remained stable over a wide range of pH, which could be attributed to the buffering nature of the substrate. This result could be significant and opens an avenue for industrial application of the pigments produced on jackfruit seed as a potent food colorant.

4. Conclusions

The most significant outcome of this study was high yield of red pigment from *M. purpureus* LPB97 using the cheap and easily available jackfruit seed. Stability of the color of the pigments over a wide range of initial pH due to the buffering nature of the substrate opens wide scope of the use of this pigment in food applications. The process of preparation of pigments is also simple and cheap. Since seeds are popular ingredients in many culinary preparations, dried and pulverized, fermented jackfruit seed powder itself could be used as a food colorant.

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