ISSN 1330-9862
(FTB-1569)

original scientific paper

Jackfruit Seed – A Novel Substrate for the Production of *Monascus* Pigments through Solid-State Fermentation

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Received: October 25, 2005
Accepted: January 19, 2006

Summary

Solid-state fermentation was carried out using jackfruit seed powder as substrate for the production of pigments using a fungal culture of *Monascus purpureus*. Due to the buffering nature of jackfruit seed powder, colour of pigments produced was stable over a wide range of initial pH of the substrate. Jackfruit seed powder with a particle size between 0.4 and 0.6 mm without any additional carbon source was found to be the best for pigment production. Water-soluble pigments were produced when jackfruit seed powder was supplemented with monosodium glutamate, soybean meal, peptone or chitin powder. The addition of external nitrogenous compounds showed a positive impact on water-soluble pigment production.

Key words: jackfruit seed powder, *Monascus*, pigment, solid-state fermentation

Introduction

Recent increasing concern regarding the use of edible colouring agents has banned various synthetic colouring agents, which have a potential of carcinogenicity and teratogenicity. Nowadays there is an increasing tendency in food industry towards natural food colours. It has long been known that microorganisms of the genus *Monascus* produce red pigments, which can be used for colouring foods. *Monascus* pigments are a group of fungal secondary metabolites called azaphilones, which have similar molecular structures as well as similar chemical properties. These pigments are produced mainly in the cell-bound state. A few examples are the orange pigments such as monascorubrin and rubropunctatin, which possess the oxolactone ring, the red pigments such as monascorubramine and rubropunctamine, which are the nitrogen analogues of the orange pigments and the yellow pigments such as monascin and ankaflavin (*1*). The pigments can easily react with amino group containing compounds in the medium such as proteins, amino acids or nucleic acids to form water-soluble pigments (*2*). Many of the studies involving *Monascus* have dealt with the general culture conditions to improve pigment production. *Monascus* is probably a xerophilic fungus, which grows in a wide variety of natural substrates (*3*). Some natural substrates that have already been tested, besides rice and other cereals, are cassava starch (*4,5*), wheat bran, wheat meal, bread meal, corn meal (*6*) and dairy milk (*7*). Currently, several companies are selling dry, pulverized, fermented rice product as a food colour and as a nutrient supplement with ability to reduce cholesterol levels, and others sell the dried product or purified extracts as food colours.

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 that could replace the synthetic ones. Generally, pigment production in industrial scale has been carried out using submerged fermentation (SmF). However, solid-state fermentation (SSF) systems appear promising due to the natural potential and advantages they offer (*8*). From the literature it is evident that utilization

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of a cheaply available substrate through solid-state fermentation can attain the objective of pigment production in an economically feasible way. Various agroindustrial residues such as rice bran, wheat bran, cassava, *etc.* have been exploited for pigment production. However, no effort has been made so far to utilize jackfruit seed as a substrate for pigment production.

Jackfruit (*Atrocarpus heterophyllus* Lam.) is a monoecious evergreen tree that is popular in several tropical countries. It is also called *jak-fruit*, *jak*, and *jaca* in Malaysia; *nangka* in the Philippines; *khanun* in Thailand; *khnor* in Cambodia; *mak mi* or *may mi* in Laos; and *mit* in Vietnam. It is an excellent example of a food prized in some areas of the world and allowed to go to waste in others. Largest of all tree-borne fruits, the jackfruit can be 8 in to 3 ft (20–90 cm) long and 6 to 20 in (15–50 cm) wide, and its mass ranges from 10 to 60 or even as much as 110 lbs (4.5–20 or 50 kg) (*9*). There may be 100 or up to 300 seeds in a single fruit. Seeds make up around 10 to 15 % of the total fruit mass and have high carbohydrate and protein contents (*10,11*). Seeds are normally discarded or steamed and eaten as a snack or used in some local dishes.

Thus, the main objective of this study was to investigate the potential of jackfruit seed powder as a substrate for *Monascus* pigment production by solid-state fermentation process.

Materials and Methods

Culture

A culture of *Monascus purpureus* LPB 97 obtained from Laboratory of Process Biotechnology, Brazil, was used in the present study. It was maintained on yeast extract--peptone-glucose medium, preserved at 4 °C and subcultured once in every three weeks.

Inoculum preparation

M. purpureus LPB 97 was grown on YPG slants 30 °C under static conditions. To fully sporulated (6-day--old) agar slope culture, 10 mL of sterile distilled water was added and the spores were scraped under strict aseptic conditions. The spore suspension obtained was used as the inoculum ($1.5 \cdot 10^5$ spores per mL).

Solid-state fermentation (SSF)

Jackfruit seeds obtained from a local market in Trivandrum were used as substrate. After peeling off the white arils of seeds (seed coats), the seeds were sliced into thin chips, dried at 60 °C for 12 h and then ground. A mass of 5 g of seed powder was taken into 250-mL Erlenmeyer flask and a salt solution (2 mL) containing (in g/L): KH_2PO_4 2, NH_4NO_3 5, NaCl 1, and $MgSO_4 \cdot 7H_2O$ 1 was added. Initial moisture was set at 65 % by adding the requisite amount of distilled water. The contents of the flasks were mixed thoroughly, autoclaved at 121 °C for 20 min and cooled to room temperature. It was inoculated with the spore suspension containing $1.5 \cdot 10^5$ spores per mL of *M. purpureus* LPB 97 and incubated at 30 °C with 50 % humidity for 7 days. Unless otherwise mentioned, these conditions were maintained throughout the experiment. Experiments were carried out to evaluate the impact of particle size of the substrate and initial pH of the substrate on pigment production. Initial pH of the substrate was achieved by adjusting the pH of the salt solution. Studies were also performed to evaluate the influence of the addition of different carbon sources (dextrose, mannitol, lactose, cassava starch, xylose, rice, sorbitol and sucrose, at 4 and 8 % by mass) and organic nitrogen sources (chitin powder, monosodium glutamate, corn steep solid, malt extract, tryptone, yeast extract, soybean meal and peptone, 1 % by mass) on growth and pigment production.

Pigment extraction

A known amount of fermented matter was taken in a 250-mL conical flask and mixed with 90 % ethanol (adding 5 mL of ethanol per gram of fermented matter on dry mass basis). The content was mixed on a rotary shaker at 200 rpm for 1 h, allowed to stand for 15 min and filtered through Whatman No. 1 filter paper. Ethanol extract of unfermented substrate was kept as the blank for pigment analysis so that any coloured substance from jackfruit seed powder was subtracted from the pigment produced by the fungus.

Pigment estimation

The analysis of pigment production was done by measuring absorbance maxima of pigment extract by spectral analysis using a double beam spectrophotometer (Shimadzu, UV 1601), taking into consideration the dilution factor of the sample (*12,13*). Only extracellular pigments were considered in this study. Pigment yield was expressed as A_{max} at corresponding wavelenght (λ) per gram of dry substrate (g) (*14*).

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 (*15*). Acid hydrolysed sample (1 mL) was mixed with 1 mL of 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 absorbance was read at 530 nm against the reagent blank. *N*-acetyl glucosamine (Sigma) was used as the standard. When the media contained a chitin supplement as nitrogen source, unfermented substrate with chitin supplementation was kept as a control and this was deduced from the estimated biomass.

TLC of the pigment extract

Crude extract was analysed by thin layer chromatography (TLC). Concentrated ethanol extracts were applied to Silica Gel 60 plates (Merck, Darmstadt, Germany) and developed with a solution of chloroform/methanol/water (volume ratio of 90:25:4) to compare the R_f values of the pigments.

Results and Discussion

The obtained results show that jackfruit seed powder served as a good substrate for the growth of *Monascus purpureus*, which resulted in a considerable amount of pigment production. The results reported are the average of three sets of experiment values and standard deviation <±5 %.

Effect of initial pH of the substrate

Irrespective of the set value of initial pH of the medium using salt solution of varied pH, a change was noticed after autoclaving the solid medium. This clearly showed the buffering nature of the substrate (jackfruit seed powder), which itself had a pH of 6.5 (Table 1).

Table 1. Optimization of pH of the salt solution in order to get the desired pH after autoclaving

pH of the salt	pH of the substrate after autoclaving
0.3	2
0.5	2.5
0.6	3
0.7	3.5
0.9	4
1.5	4.5
1.7	5
8.4	5.5
11.5	6
12.3	6.5
12.9	7
13.2	7.5

There are reports in the literature describing the buffering nature of different agroindustrial residues (*16*). Set initial pH values of the substrates were between 2.0–11.0. At very low initial pH substrate (2.0 and 2.5), there was no fungal growth (Fig. 1). From the spectral analysis it was observed that for pH=3.0, the absorbance maximum

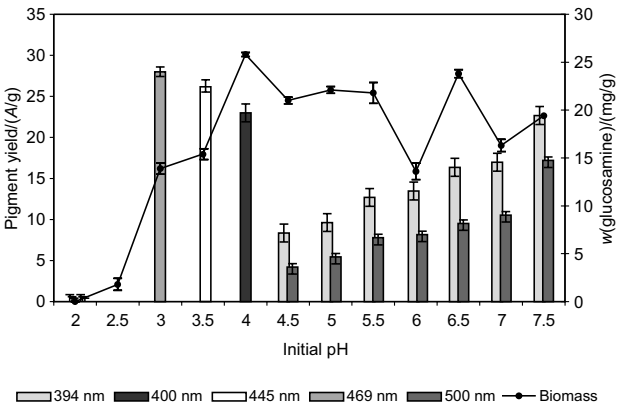

Fig. 1. Effect of initial pH of the substrate on growth and pigment yield

was obtained at λ=469 nm, which corresponded to orange pigments. For pH=3.5 and 4.0, the A_{max} shifted towards lower wavelength, giving the peak values at 445 and 394 nm, respectively. Over a wide range of pH, *i.e.* from 4.5 to 7.5, pigment yield showed similar absorption peaks around 390 and 500 nm, although with varied pigment production. These results were confirmed by separating the pigment extracts by TLC (Fig. 2). Spots I, II and III indicate the respective R_f values of

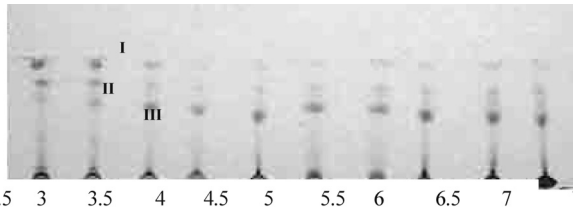

Fig. 2. TLC of pigment extracted from fermented substrate of different initial pH

yellow, orange and red for the pigment extracts. R_f values of spots I, II and III were essentially the same for all extracts, ranging from pH=4.5 to 7.5. These results indicated that the same yellow, orange and red components were produced during fermentation, regardless of the initial pH of the substrate. Contaminants were not identified on TLC plates, since the spots obtained were in agreement with the results of spectral analysis. This could be attributed to the buffering capacity of jackfruit seed powder. Since jackfruit seeds are a popular ingredient in many culinary preparations, the dried, pulverized and fermented seed powder itself could be used as a food colorant. Furthermore, the process involved in the fermentation was simple and cheap. These results could be quite significant as they open the scope of jackfruit seed powder (which is otherwise discarded as waste) as a potent food colorant by bringing down the production cost of these food grade pigments.

Effect of particle size

Among the several factors in SSF processes which are important for microbial growth and activity, the substrate particle size is one of the most critical parameters (*17,18*). Generally, smaller substrate particles provide a larger surface area for microbial attack, and thus it should be considered as a desirable factor. However, too small particles may result in substrate agglomeration, which may interfere with aeration (due to less interparticle space) and may, thus, result in poor microbial growth. At the same time, larger particles provide better aeration efficiency (due to increased interparticle space), but provide limited surface for microbial attack. Therefore, it may be necessary to provide compromised particle size (*19*). In the present study, jackfruit seed powder of different particle sizes was used to prepare different media, *viz*. M_1 (particles <0.09), M_2 (particles between 0.09 and 0.1 mm), M_3 (particles between 0.1 and 0.2 mm), M_4 (particles between 0.2 and 0.3 mm), M_5 (particles between 0.3 and 0.4) and M_6 (particle between 0.4 and 0.6 mm). Substrate moisture was set at 65 %. Results indi-

cated that particles between 0.4 and 0.6 mm were optimal for pigment production (Fig. 3). In the subsequent experiments, therefore, particle size between 0.4 and 0.6 mm of jackfruit seed powder was used for the production of pigments.

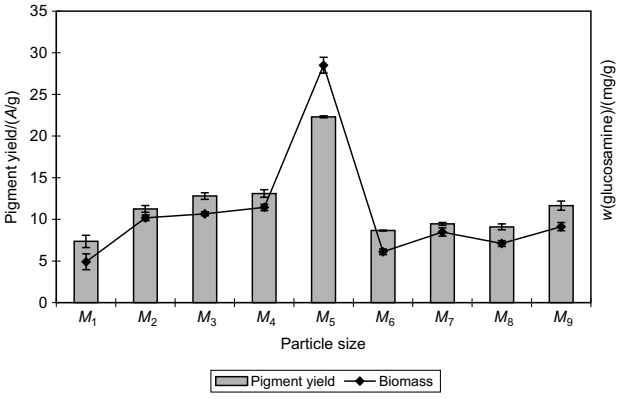

Fig. 3. Effect of particle size on growth and pigment yield

Effect of supplementation of carbon source

Jackfruit seed powder medium was supplemented with additional carbon source at two different concentrations (4 and 8 % by mass). In our study, at 4 %, even though dextrose supported the growth of the organism, pigment yield was very negligible when compared to other carbon sources. Control itself gave the maximum yield for red pigment (19.5 A/g) as well as yellow pigments (19 A/g), followed by rice supplementation with a pigment yield of 18 A/g for red and 18.3 A/g for yellow. Even though there was an increase in the growth, pigment yield was poor when supplemented with sorbitol and mannitol. Lactose, cassava starch and sucrose were found to be good supporters for pigment production next to rice and control, except for sucrose where there was a reduction in growth (Fig. 4). A significant observation from the spectral analysis (Fig. 5) was that there was a shift in absorbance maxima for xylose, giving peak values at 498 and 409 nm. Even though it did not support growth as well as other supplements, the yield of pigment with an absorbance peak at 409 nm was close to other best carbon sources. Utilization of carbon sources for growth appears to be strain specific since for other stains of *Monascus* glucose and its oligo- and polysaccharides were better than other carbon sources both for growth and pigment production (*20*–22).

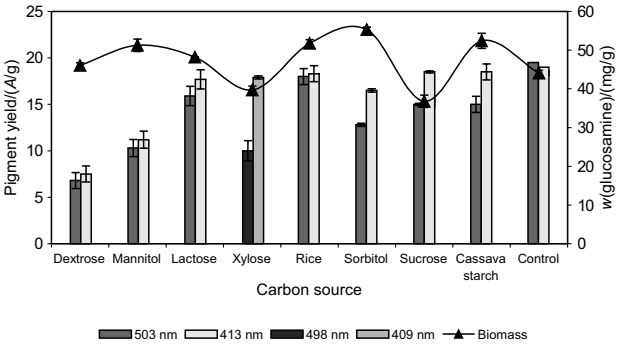

Fig. 5. Effect of supplementation of carbon source (4 % by mass) on growth and pigment yield

At 8 % (Fig. 6), there was an inhibition in pigment production for those samples supplemented with dextrose, mannitol and lactose. Rice supplementation gave only a marginal increase in pigment yield (*19.5* A/g for red pigment and 22.5 A/g for yellow pigment) when compared to the control (17.9 A/g and 20.8 A/g for red and yellow pigments, respectively). Regarding yellow pigments, xylose gave the maximum yield (23.3 A/g) and it should be noted that at 8 % xylose supplementation resulted in the production of only yellow pigments with a single absorbance peak at 413 nm (Fig. 7). Therefore, considering both red and yellow pigments,

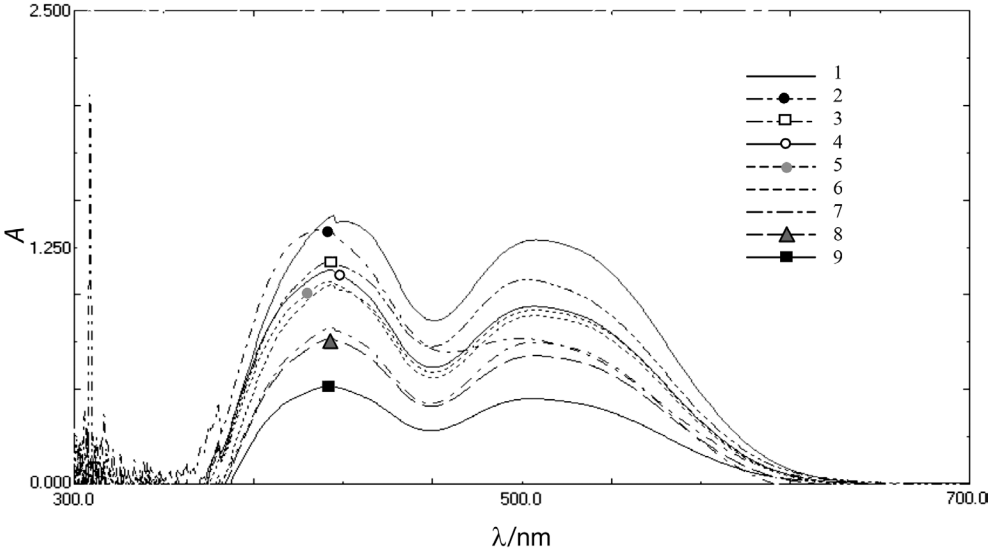

Fig. 4. Spectrum of pigment extracts showing the effect of additional carbon source (4 % by mass)
1 jackfruit seed powder without carbon source, 2 xylose, 3 rice, 4 lactose, 5 sucrose, 6 cassava starch, 7 sorbitol, 8 mannitol, 9 dextrose

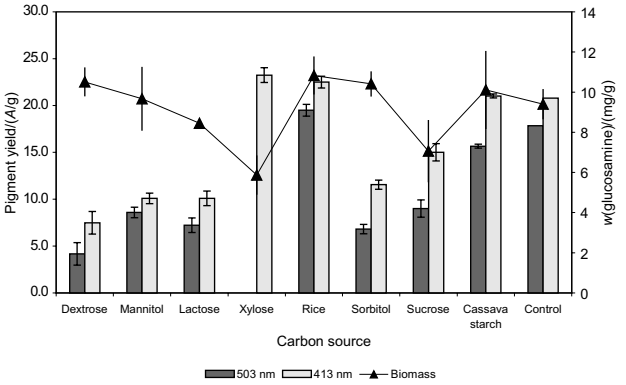

Fig. 6. Effect of supplementation of carbon source (8 % by mass) on growth and pigment yield

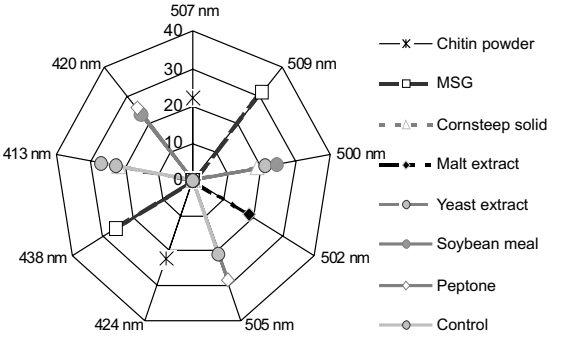

Fig. 8. Effect of supplementation of organic nitrogen source on pigment yield

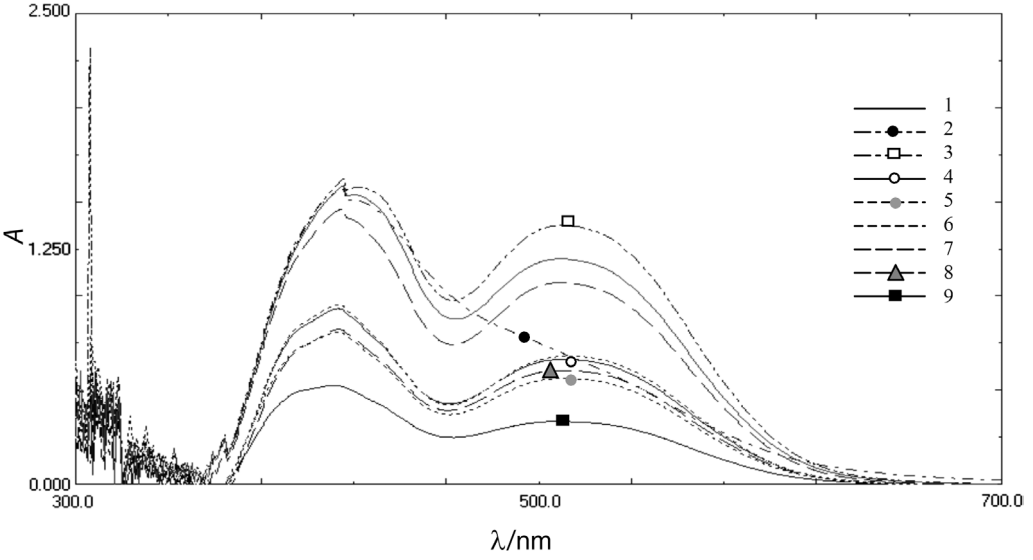

Fig. 7. Spectrum of pigment extracts showing the effect of additional carbon source (8 % by mass)
1 jackfruit seed powder without carbon source, 2 xylose, 3 rice, 4 lactose, 5 sucrose, 6 cassava starch, 7 sorbitol, 8 mannitol, 9 dextrose

and since only marginal increase in pigment yield was obtained for the best carbon source at higher concentration, jackfruit seed powder without any supplementation of carbon source can be utilized as a suitable substrate for pigment production.

Effect of supplementation of nitrogen source

Utilization of different nitrogen sources has been known to produce different pH patterns in fermentation, which affects growth and pigment production (*23*,*24*). But in our study, difference in pigment yield cannot be attributed to different pH pattern due to buffering nature of jackfruit seed powder. From the spectral analysis, slight shift in absorbance maxima was observed for different organic nitrogen sources. In our study monosodium glutamate was found to be outstanding for both red and yellow pigment production (30.8 and 25.5 A/g, respectively), followed by peptone, soybean meal and chitin powder (Fig. 8). This result was similar to the findings of Lin and Demain (*21*,*22*) who reported that monosodium glutamate was most suitable for pigment production. They observed that organic nitrogen was optimal for growth, but unfavourable for pigment production. Spectral analysis of water extract showed that jackfruit seed powder without any addition of nitrogen source was not able to produce any water-soluble pigments. It has been reported that the addition of monosodium glutamate could give rise to water-soluble red pigments (*25*). In the present study, the addition of nitrogen sources such as monosodium glutamate, soybean meal, peptone and chitin powder gave water-soluble pigments with maximum absorbance peaks at 484 and 413 nm, 482 and 405 nm, 482 and 402 nm, 484 and 385 nm, respectively (Fig. 9), whereas only single absorbance peak corresponding to yellow pigment was obtained when supplemented with corn steep solid, malt extract and yeast extract. Although the monascorubrin-rubropunctatin mixture, which constitutes the orange pigment produced as the direct fermentation product of *Monascus* species, is water-insoluble and therefore of limited utility as a food colorant, it has been reported that these materials react with primary amines to afford red colorants, many of which are water-soluble (*26*). Therefore, jackfruit seed powder supplemented with required organic nitrogen sources could be considered as a poten-

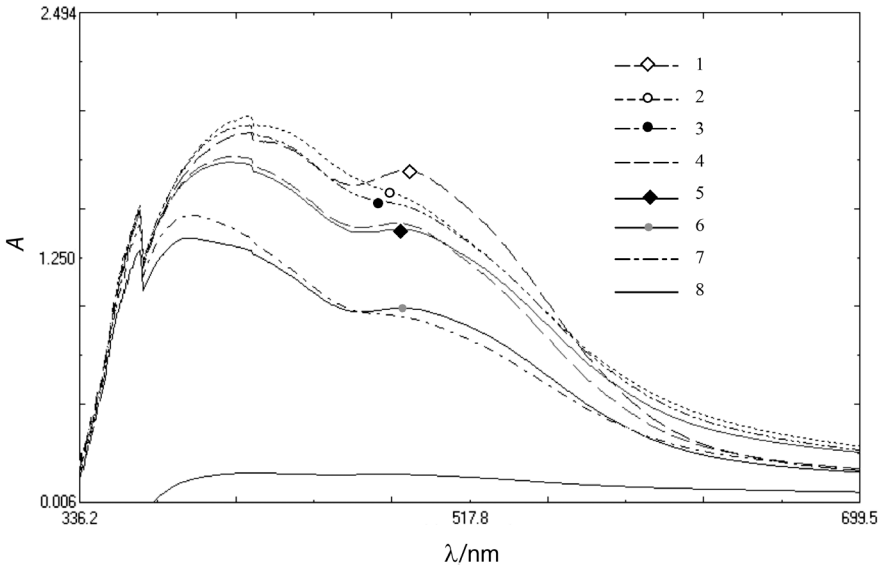

Fig. 9. Spectrum of water extract showing the effect of additional organic nitrogen
1 monosodium glutamate, 2 corn steep solid, 3 yeast extract, 4 peptone, 5 soybean meal, 6 chitin powder, 7 malt extract, 8 control

tial substrate for the production of water-soluble *Monascus* pigments.

Conclusions

From the results it could be concluded that jackfruit seed could be an effective substrate for the production of pigments by fungal culture of *Monascus* sp. The fungal culture did not require any additional carbon source, but supplementation of external nitrogen sources was useful in enhancing the pigment production, especially water-soluble pigments. It could also be established that by varying the fermentation conditions, the fungal metabolism changed to produce yellow or red pigments in varying concentrations, which could be significant for industrial application.

Acknowledgements

SB acknowledges the award of a fellowship by the Council of Scientific and Industrial Research, Government of India, New Delhi, India.

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