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A Solid Liquid State Culture Method to Stimulate Monascus Pigments by Intervention of Different Substrates

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Abstract

The present study illustrates the investigation carried out on the production of pigment by Monascus sanguineus and Monascus purpureus MTCC 410 under solid state and submerged conditions. Also, in addition to that, various aspects of pigments, the effect of physical and nutritional factors and different substrates for enhanced pigment production were studied. Growth pattern of M. purpureus and M. sanguineus was studied in tryptone glucose yeast extract and it was found that maximum biomass and pigment yield was noticed on 15th day of incubation in this growth medium and dry weight obtained was 2.25g/l. Screening of substrates for both the sp. was also carried out. Both sp. were able to grow in all the experimented substrates. Coconut residue was found to be the best substrate for Monascus purpureus MTCC 410 whereas Potato peel was recognized to be the best substrate for Monascus sanguineus. Different polar and non polar solvent were used for extraction of pigment and among them methanol was found to be best solvent for extraction of red pigment.

Keywords: Solvent, substrate, coconut residue, methanol.

Introduction

Monascus strain is a homothallic fungus. This fungus is characterised by its ability to produce secondary metabolites of polyketide structure synthesized by the polymerization of acetyl and propionyl subunits by a process similar to the fatty acid synthesis[1]. The type of carbon sources and the composition of the starch directly affect the growth of *Monascus* species[2]. Aldohexoses such as dextrose and glucose are considered to be relatively better carbon sources for growth of *M. purpureus* than sugar alcohols such as mannitol and sorbitol, while sucrose reduced the growth of the fungus[3]. The colour of the pigment is influenced by the culture conditions, pH value and the carbon and nitrogen sources in the substrate[4,5]. Angkak,a conventional Chinese functional food produced through solid state fermentation generally on cooked rice with *Monascus* sp., which known to produce various high value secondary metabolites such as lovastatin, γ-aminobutyric acids (GABA), monascodilone, monascorubramine, ankaflavin, monascin, rubropunctatin[6,7]. Lovastatin is one of the most studied secondary metabolites of angkak and is considered to be a competitive inhibitor of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, which is a rate limiting and regulatory enzyme of cholesterol biosynthesis[8,9].

From the literature, it is clear that exploitation of an inexpensively available substrate through solid-state fermentation (SSF) could accomplish such an objective. In SSF method , the solid substrates not only serve as an anchorage for the cells , but also supply the nutrients to the microbial culture budding in it [10].

The objective of the present study was to find out optimum condition for pigment production under solid liquid state culture condition by *Monascus* strains. For solid state fermentation, cheap agro-waste has been used for pigment production. Both *Monascus* strain *(Monascus purpureus and Monascus sanguineus)* were able to utilize all experimented substrates and had produced pigment. Extraction of pigments has been done with both polar and non polar solvents and marked difference seen in pigment yield.

Material and Methods

Source of reference culture: Monascus purpureus MTCC 410 used as reference strain was obtained from the Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India.

Isolated Culture: Wild strain of *Monascus* was isolated from pomegranate (Punica granatum) and identified as *Monascus sanguineus* and maintained on Potato Dextrose Agar (PDA) medium. It was incubated at 30°C for 7 days, preserved at 4°C[11].

Inoculum preparation: Approximately, one loop of spores from agar slope was diluted with distilled water. This spore's suspension was inoculated in 100 ml PDB broth and incubated in shaker at 30°C at 120 rpm for five days for both the strains[12].

Growth Pattern of Monascus strains; pH, temperature and incubation time: Tryptone glucose broth as a media was selected for investigation of growth pattern for both the strains. 50 ml of the above media was autoclaved in 100 ml flask. These media were inoculated with 0.5 ml of culture from both the

strains separately. Biomass and pigment were estimated every 5th, 7th, 9th, 11th, 13th and 15th day after inoculation. The same course of action was applied to investigate the effect of temperature and pH on pigment production and the mycelial growth for both strains. To investigate the influence of temperature, the cultures were incubated at 16, 30, 37, and 50°C. The effect of pH was studied at pH range from 3 to 9. After inoculation these flask were kept in static condition for 15 days[13].

Effect of carbon and nitrogen sources on production of pigment in submerge culture: Effect of different carbon and nitrogen sources on pigment production and biomass was investigated in broth for both strains. Carbon and nitrogen ratio used was 10:1. Glucose, Fructose, Lactose, Maltose, Raffinose and Starch were utilized as carbon sources. Peptone, yeast extract, tryptophane and asparagine were used as a nitrogen sources. Five gram of each carbon sources supplemented with 0.5g of each nitrogen sources were placed separately in a 100 ml conical flask to which 50ml of distilled water was added. The pH of the medium was maintained at 6 and autoclaved for 20 minutes at 121°C. The medium was inoculated separately with 10% of the seed culture from both the strains. These flasks were incubated at 28°C in static condition for 10 days[14].

Dry cell weight: The mycelia were separated from the broth by filtration (Whatmann No. 1) for both the strains and were washed with distilled water. This was then dried in an oven at 50°C. The results were presented in grams per liter[15].

Pigment estimation: For the estimation of the pigment the filtrate was centrifuged at 10000 *g* for 15 min. Pigment concentration was determined with the help of colorimeter at 510 nm[16].

Solid-state fermentation (SSF) for pigment production: For SSF, three substrates viz. orange peel, potato peel and coconut cake were chosen. Five gram of above substrates along with distilled water was placed in a 100 ml conical flask. The pH of the medium was adjusted to 6. It was then autoclaved for 20 minutes at 121°C. These substrates were inoculated with 10% of the seed culture from both the strains separately. These substrates were incubated with 56-60% relative humidity at 28°C for 20 days[11].

Effect of nitrogen and carbon source on pigment production in solid state fermentation: Above substrates were supplemented with different nitrogen sources such as soyabean and ammonium sulphate at 1% w/w and carbon sources such as glucose, fructose and glycerol at 5% w/w concentration separately for pigment production. The substrate (5g) was then supplemented with these concentrations of nitrogen and carbon sources used in the experiments according to the procedure described above[17,18].

Pigment extraction and estimation for solid substrate: The culture medium was dried for 48 hours at 50°C. One gram of solid substrate (fermented) was taken for extraction of the pigment using 20 ml of 95% ethanol on a rotary shaker for 24 hrs at 200 rpm. Pigment absorbance was done with colorimeter at 510 nm[19].

Extraction of pigment using different solvents: Extraction of pigment of *Monascus purpureus* and *Monascus sanguineus* was also done with different solvents viz. non polar to polar. Benzene, chloroform, ethyl acetate, methanol and water were used for the extraction[20].

Results and Discussion

Growth Pattern of Monascus strains; pH, temperature and incubation time: The result showed that *Monascus purpureus* was able to grow rapidly with time on this media but, *Monascus sanguineus* has shown poor pigmentation but satisfactory mycelia growth. Maximum biomass and pigment was observed on 15th day of incubation for both strains (For *Monascus purpureus* dry weight of 2.4g/l and 3.75 O.D Units/ml and for *Monascus sanguineus* dry weight of 2.0 g/l and 2.25 O.D Units/ml) (figure 1). Optimum temperature was observed at 30°C for pigment production and mycelial growth for both the strains (figure 2).

The results for pH variation showed that maximum biomass and pigment yield was observed at acidic pH (4) for both the strains and the yellow and orange pigments were observed at this pH simultaneously. The growth of both *Monascus* strains was noticed at entire tested range of pH (3 to 9) (figure 3).

Nimnoi and Lumyong[18] have concluded tryptone glucose yeast broth (TGY) as a good medium for the growth and pigment production by *Monascus* strain.

The temperature of incubation has got profound influence on the cell synthesis and pigment production. In the present study, the different temperatures viz., 15, 28, 37 and 55°C were set for *Monascus* pigment production. At 15°C and 55°C, the accumulation of pigments in media was markedly affected. The temperature and the pigment production were negatively correlated. The optimum temperature for higher biomass and pigment production was 28°C, as this temperature may favour utilisation of nutrients that leads to higher microbial biomass and pigment production. Other components in medium could interfere with production of red pigments and the biomass[21,] Chen and Johns[22] explored that the fungal growth and ankaflavin (pigment) synthesis were better at low pH. The physiology of fungi, pigment synthesis and conidial development is influenced by different pH levels. At pH 4.5 (acidic range), the conidiation was found to be escalating while the red pigment synthesis showed reduction[16].

Effect of carbon and nitrogen sources on production of pigment in submerge culture: Results showed that for *M. purpureus*, glucose supplemented with peptone strongly stimulated both the growth and pigment production (1.65 OD

Units/ml) whereas for *M. sanguineus*, maximum yield (1.74 OD Units/ml) was observed with media supplemented with glucose and yeast extract. As far as the amino acids are concerned, it was seen that both the species gave highest pigment yield in the medium containing fructose and tryptophan (1.64 OD Units/ml for *M. purpureus* and 0.9 OD Units/ml for *M. sanguineus*) (figure 4a and b). Findings from this report indicate that asparagine can promote biomass production and yellowish-orange pigments, but this amino acid is not suitable for red pigment production. *Monascus* pigments are amino acid derivatives. Amino acids are showing infuence in the biosynthesis of red pigments.

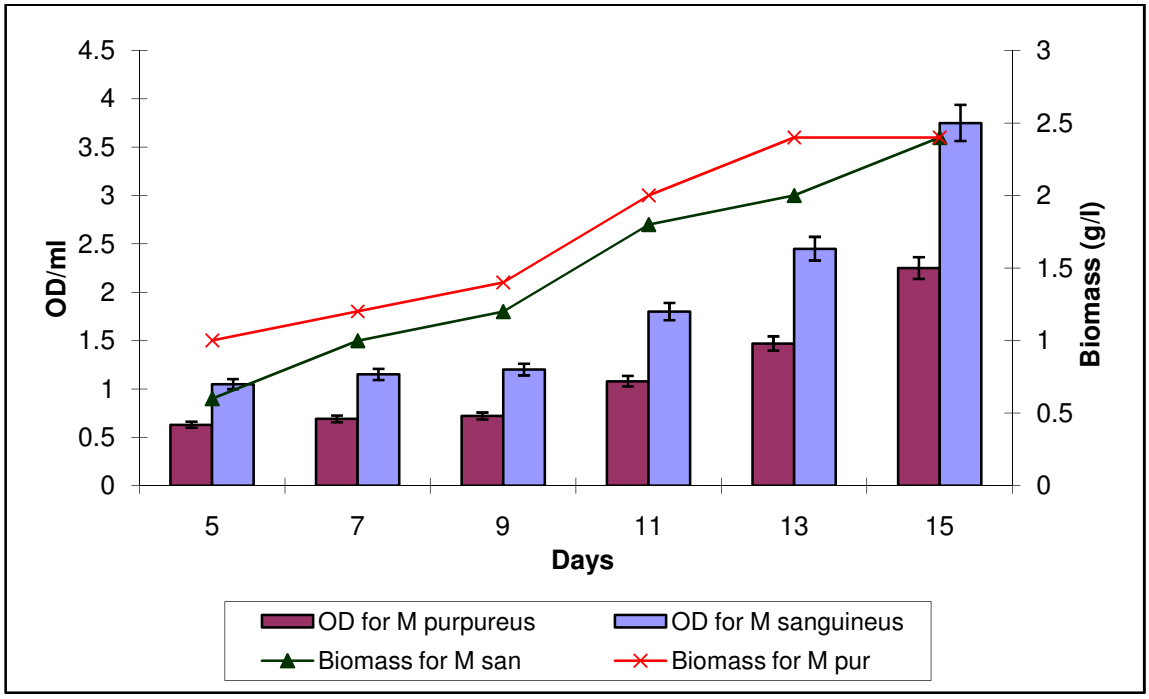

Figure-1
Growth pattern of *M. sanguineus* and *M. purpureus* on TGY

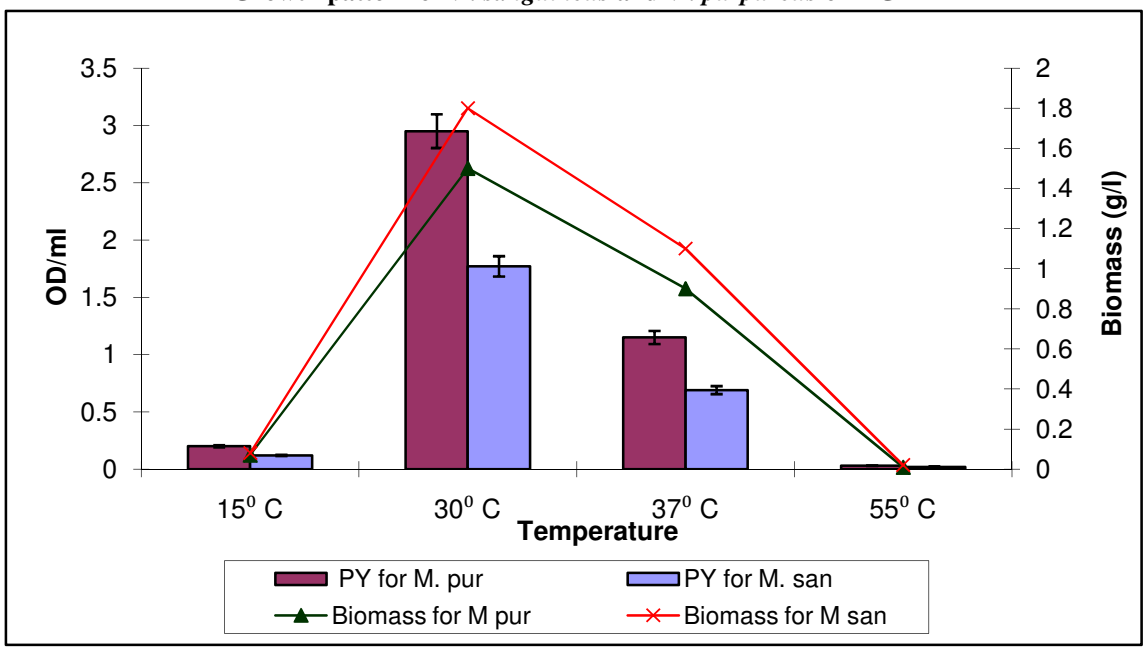

Figure-2
Effect of temperature on pigment yield and biomass of *M. purpureus* and *M. sanguineus*

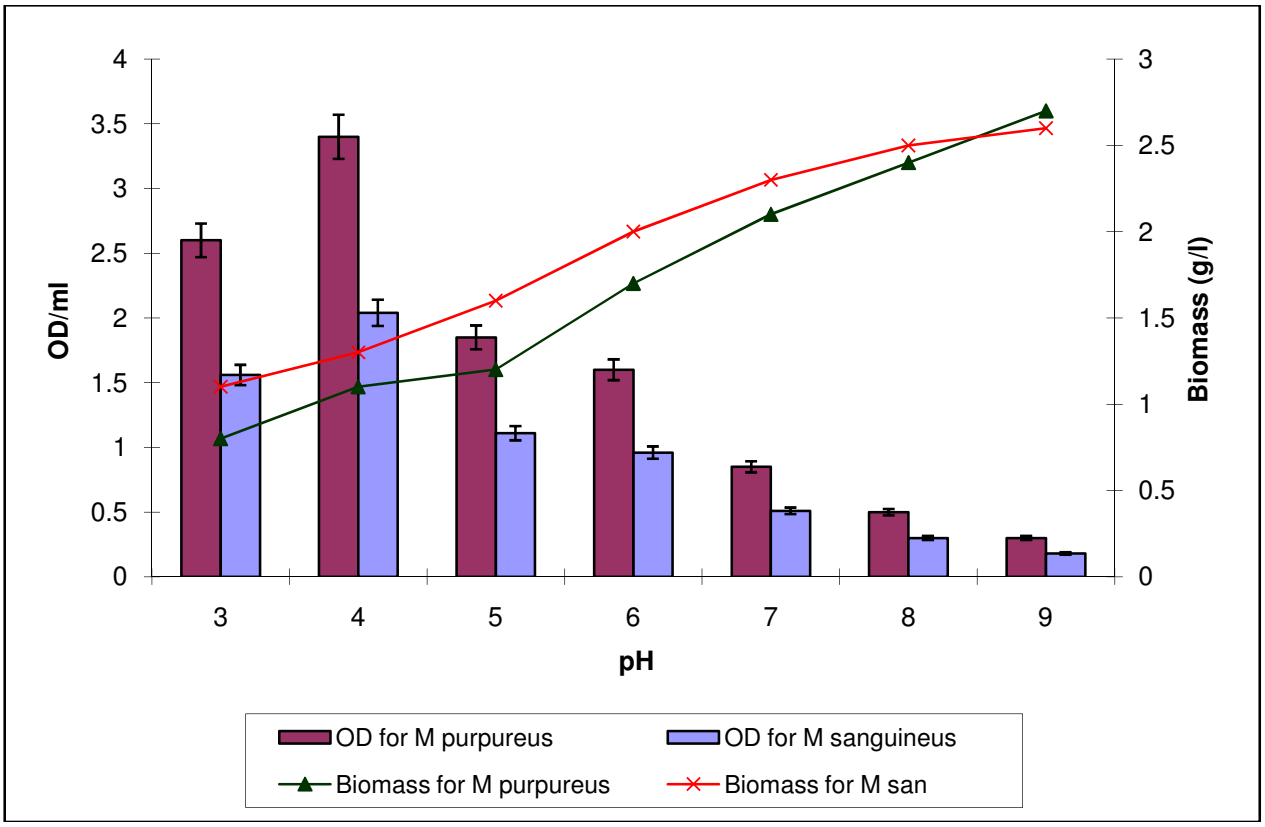

Figure-3
Effect of pH on pigment yield and biomass of *M. purpureus* and *M. sanguineus*

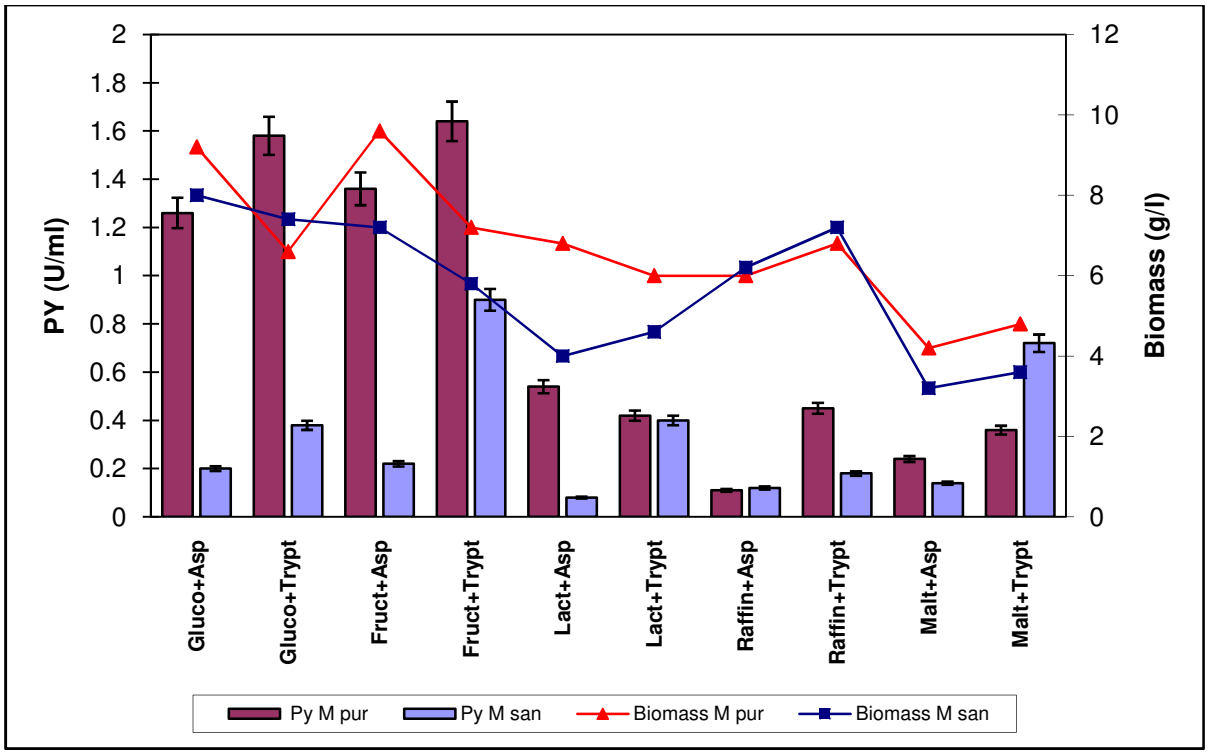

Figure-4(a)
Effect of carbon amino acid source on pigment production

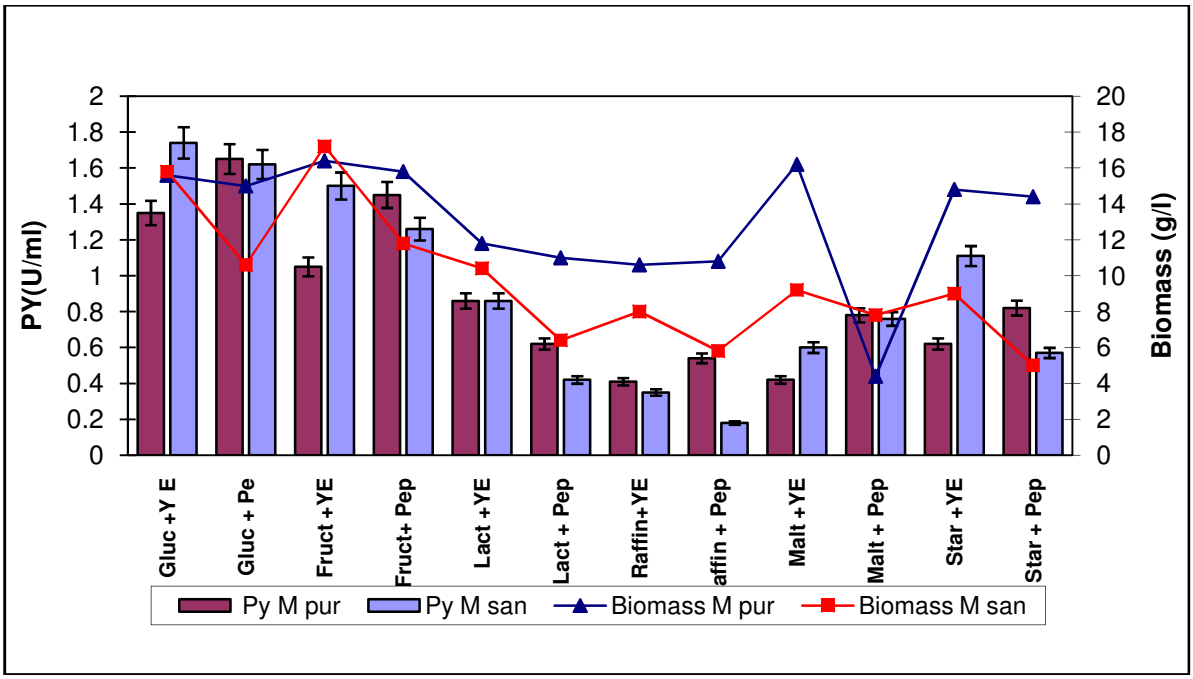

Figure-4(b)
Effect of carbon nitrogen source on pigment production

The nature of the nitrogen source affected pigment production as well as the fungal growth, independent of pH. Ammonium and peptone as nitrogen sources was found good for growth and pigment production[22].

Hajjaj et al.[23] has reported that *Monascus ruber* ATCC 96218 cultivated on chemically defined media and when glycine, tyrosine, arginine, serine, or histidine were used as sole nitrogen sources, they favoured the production of red pigments, and restricted the synthesis of the mycotoxin.

Screening of substrates for both strains: It was found that both strains *Monascus sanguineus* and *Monascus purpureus* MTCC 410 grew on all experimented substrates though pigment yield varied. For *M. purpureus*, coconut cake with 0.73 O.D Units/gds at 510 nm showed maximum pigment yield followed by orange peel with pigment yield of 0.65 O.D Units/gds at 510 nm. For *Monascus sanguineus*, potato peel with 0.68 O.D Units/gds at 510 nm showed maximum pigment yield followed by coconut cake with 0.56 O.D Units/gds at 510 nm. (figure 5). SSF possesses several biological advantages when compared with submerged fermentations. Such advantages include higher fermentation productivity, less catabolic repression, low water demand and hence, lower sterility demand due to the low water activity, cultivation of microorganisms requiring a solid support, and mixed cultivation of *Monascus*[24].

Effect of Carbon and Nitrogen Source on production of pigment by Monascus strains: For *M. sanguineus,* highest pigment yield was seen when the substrates were supplemented with glycerol. Potato peel (2.62 O.D Units/gds) showed maximum pigment yield followed by coconut cake 1.82 O.D Units/gds) and orange peel (1.62 O.D Units/gds). For *M. purpureus* the utmost pigment yield was seen when coconut cake was supplemented with glycerol (with pigment yield 2.85 O.D Units/gds) and pigment yield was maximum when orange peel (with pigment yield 1.47 O.D Units/gds) and potato peel (with pigment yield 2.76 O.D Units/gds) were supplemented with fructose (figure 6).

Both *Monascus* strains were able to grow in both organic and inorganic tested nitrogen sources. Maximum pigment yield was noticed when the substrates were supplemented with soybean.

The *Monascus* pigment is produced via a polyketide pathway, requiring acetyl Co-A, which is produced from glucose via pyruvate[4]. Pongrawee and Saisamorn[18] concluded that addition of glucose enhances pigment production. Glucose and oligosaccharides are better than any other C source for both growth and pigment production[25,10].

Shepherd and Carels[26] reported that N source affected the growth and pigment production. This is also dependent on the culture conditions and C-N ratio[16,27] demonstrated that most intense red colour was observed when RD6 (rice) as a substrate was supplemented with soybean milk.

Extraction of pigment using different solvents: The results indicated that there was maximum efficiency in extraction using methanol (for *M. purpureus*- 1.08 OD U/gds and for *M. sanguineus*- 1.68 OD U/gds) followed by water, ethyl acetate, chloroform and benzene (figure 7). Results showed that polar solvents seemed to perform better than excessive polar (water) or non-polar (benzene, chloroform) solvents. *Monascus*

pigments are polyketide and hydrophilic in nature. Methanol is an organic polar solvent and according to chemical structure these pigments are slightly polar, so they bind easily with methanol or polar molecules. Hence because of polarity of solvent, extraction of pigments was more. In non polar solvent like ethyl acetate, chloroform or benzene recovery of pigments was very less. Though water is a polar solvent, the pigment extraction was less and change in colour was also observed. Our observation indicates that water is not suitable for the extraction of pigments since it is an inorganic solvent.

Carvalho *et al.*[20] investigated the use of different solvents for the extraction of pigments and concluded that methanol was the best and water was not suitable for the extraction of *Monascus* pigments.

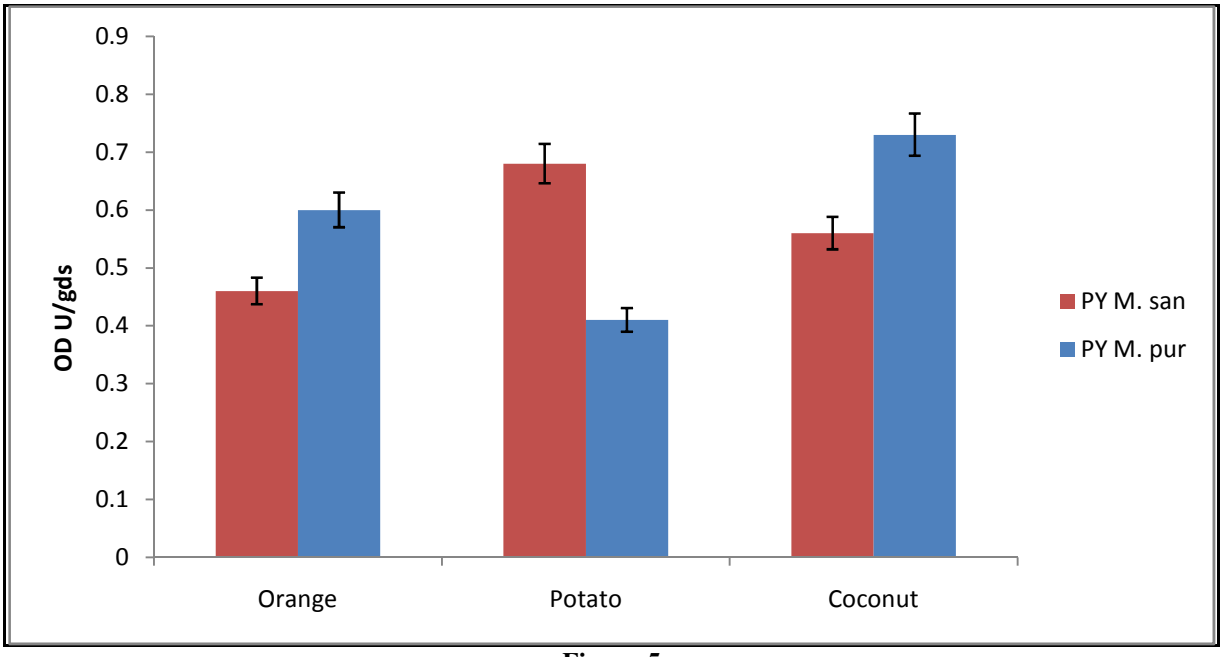

Figure-5
Screening of substrates for pigment production

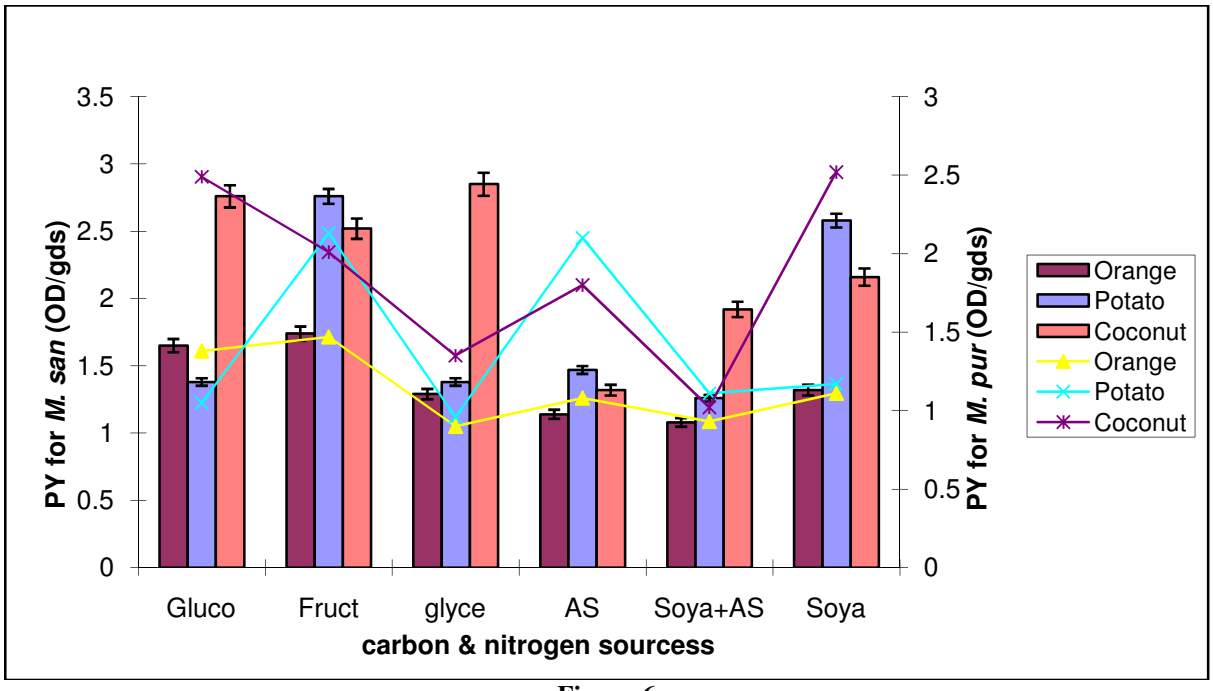

Figure-6
Effect of Carbon and Nitrogen Source on pigment yield by *Monascus* strains

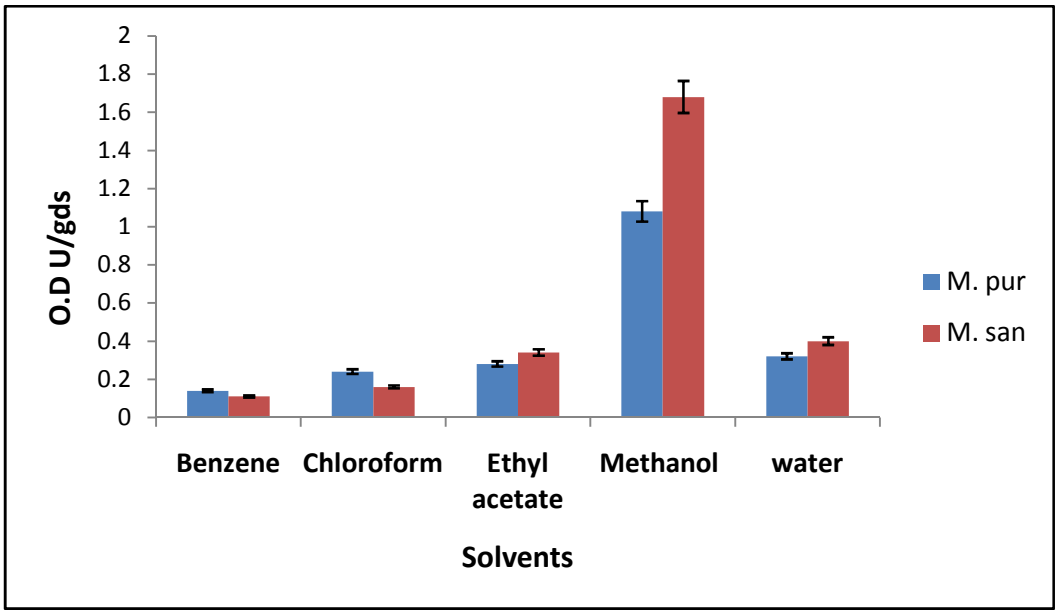

Figure-7
Extraction of pigment with different solvents

Conclusion

Optimization of pigment yield with *Monascus purpureus* and *Monascus sanguineus* was done in both liquid and solid state fermentation condition. Both *Monascus* strains were found to be an effective source for pigment production. Maximum yield of pigment was observed in polar solvent for both the *Monascus* strains

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