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BIOUTILIZATION OF KINNOW WASTE FOR THE PRODUCTION OF BIOPIGMENTS USING SUBMERGED FERMENTATION

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ABSTRACT

Biopigments are the microbial products, which have number of advantages over synthetic pigments. Fungi like *Monascus purpureus* produces pigments, which possess certain therapeutic properties, coloring properties having wide applications in food, pharmaceutical and textile industries. The present investigation was carried out for the optimization of media and process parameters for efficient biopigment production utilizing fruit waste in order to reduce cost of the process and pollution related problems. Kinnow peel waste was used as a substrate for the production of red pigment using *Monascus purpureus* MTCC 369 using submerged fermentation. Maximum pigment production was recorded when the media containing kinnow peel powder and pea pod supplemented with magnesium sulphate (0.1%) with pH of 6.5, incubated at 35 °C after 9 days of incubation period. Among the different solvents and concentration tested, maximum pigment production extraction was observed with 90% ethanol.

Keywords*: Monascus,* kinnow waste, media, biopigments, submerged fermentation.

INTRODUCTION

The interest in the natural pigments has increased in recent years due to the consumer concern about the harmful effects of synthetic pigments on health and with the development of new food products based on natural ingredients (Dufosse, 2006; Buhler *et al.,* 2013). Pigments can be derived from natural sources such as plants, insects & microbes. Now-a-days, the potential of natural colors from microbial pigment is being investigated immensely. Microbial pigments are advantageous in terms of production, when compared to pigment extracted from vegetables or animals because they have the potential of being exploited using different culture techniques for scale up of the process. Further, micro-organisms have high growth rate and yield (Babitha, 2009), which will cut down the production time and process lends itself to continuous operation (Hendry and Houghton, 1997). In addition, production becomes flexible & can be easily controlled as compared to plant or animal sources Therefore, it is great advantageous to use microbes as a producer for pigment production due to its intrinsic properties of high growth rate, no seasonal variation, high production rate and ease of manipulation (Joshi *et al.,* 2003).

Biopigments have been produced from large number of bacterial, yeast and mold species. The microorganisms to be used for pigment production should have some necessary features (Joshi *et al.,* 2003). Among different microorganisms, *Rhodotorula* sp., *Achromobacter* sp., *Blakeslea* sp. and *Monascus* sp. are common pigment producing microbes. The use of Monascus pigments in food industry has been carried out traditionally in the oriental foods for hundreds of years (Teng and Feldhein, 2001; Babitha et al., 2004). The genus *Monascus* belonging to family Monascaceae & class Ascomycetae involves three main species *M. pilosus*, *M. purpureus* & *M. ruber* for pigment production (Buhler *et al.,* 2013). The ability to produce secondary metabolites of polyketide structure is the important feature of *Monascus* species. Pigments from this fungus can be widely used in food industries & in pharmaceutical industries for therapeutic use (Kumar *et al.,* 2012). Generally yellow, orange and red pigments are produced by these species (Buhler *et al.,* 2013). It is well known that microorganisms of genus Monascus produce red pigments, which can be used for coloring food stuffs (Babitha *et.al.,* 2006). Moreover, microbial pigments possess a range of biological activities such as anti-mutagenic, anti-cancer properties, anti-microbial & anti-obesity characteristics, they could even be utilized for dyeing cotton yarn & leather (Velmurugan *et.al.,* 2010) and preparing gels (Calvo and Salvador, 2002).

Monascus species is probably a xerophilic fungus which can grow in wide variety of natural substrates (Babitha et al., 2004). Normally pigment production in industries is carried out using submerged fermentation on different substrates (Babitha *et al.,* 2006). Generally, submerged fermentation has been preferred due to low production yield in case of solid state fermentation (Kim et

al., 2002). The utilization of agro-industrial byproducts for biopigment production can make the process economically feasible.

Pigments can be easily extracted by treating the fermented dry matter with different solvents. The type of solvent and its concentration, ratio of solvent to solid and pH are important factors that influence the product extraction (Hasim, 2008). To economize the process on industrial scale, there is need to develop low cost technology for the production of pigments that could replace synthetic ones.

Kinnow (*Citrus reticulata*), which is a hybrid belongs to the citrus family, and is one of the major value added citrus fruit crops of India, which is extensively cultivated in Northern Region including Punjab, Haryana, parts of Himachal Pradesh and Rajasthan. With increasing demand and consumption of kinnow fruit, large quantities of waste is also increasing. This waste is often an economic liability to the fruit processors, as waste disposal is a growing problem. Keeping in view the above, the present investigation was carried out to study to standardize the bioprocess for the production of pigment from kinnow peel using *M. purpureus* by submerged fermentation. The optimization of media components & other process parameters to evaluate the potential for growth & pigment production was carried out. Further the effect of different solvents on pigment extraction was also evaluated to find out the best extracting solvent for pigment production.

MATERIAL AND METHODS

MICROORGANISM

Monascus purpureus MTCC 369 was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh. This culture was maintained on growth media containing: 200 g/l potato (scrubbed and diced), dextrose (20 g/l) by subculturing, aseptically at fortnight intervals and stored at 4°C, until further use.

PREPARATION OF INOCULUM

The fungal culture *M. purpureus* MTCC 369 was grown on the potato dextrose agar (PDA) slants at 30 °C under static conditions. To fully sporulated agar slope culture, 10 ml of sterile water was added & spores were scraped under strict aseptic conditions. The spore suspension thus obtained was used as inoculum.

SUBMERGED FERMENTATION

During the preparation of fermentation media for submerged fermentation, kinnow peel as carbon source was used by replacing glucose and peptone with pea pod (unless otherwise specified). The composition of the fermentation media (g/l) used for the growth of the fungal culture consisted of kinnow peel powder 5.0, peptone 0.2, potassium nitrate 0.2, ammonium phosphate 0.05, magnesium sulphate 0.1, calcium chloride 0.05 and pH was set as 6.0 unless otherwise specified. During medium optimization, the salts were used individually to replace the above mixture of salts. The media was sterilized using autoclave at 15 psi for 20 min. The seed culture was added into 100 ml growth media. The flasks were incubated at specified temperature for 9 days. During the course of fermentation, samples were taken to monitor the biopigment production.

EXTRACTION OF BIOPIGMENTS

The extraction of biopigments was carried out using different solvents according to the method of Velmurugan et al. (2010). The mycelium obtained was separated from the broth by filtration using Whatman paper 1, and was crushed in a pestle & mortar. Different solvents (ethanol, methanol, Dimethylsulphoxide) were added to 1 g of the fermented biomass for extraction of pigment. The mixture was kept on rotary shaker for 1 hour and the upper layer containing pigment was collected.

PIGMENT ESTIMATION

Pigment concentration was determined by taking absorbance at specified wave-length of 500nm using UV-VIS spectrophotometer (DR 5000, HACH, Germany). The absorbance values were converted into color value unit by using formula as described by Dikshit and Tallapragada (2011).

ESTIMATION OF BIOMASS

The fungal biomass in the synthetic medium was filtered through pre-weighed Whatman filter paper and washed twice with deionized water followed by drying at 105 °C for 12-15 hrs and weighed to estimate the biomass yield (Velmurugan *et.al.,* 2010).

RESULTS AND DISCUSSION

Designing a fermentation medium is a critical process as the medium composition can significantly affect the product yield (Kennedy and Krouse, 1999; Panda et al., 2007). An optimally balanced culture medium is necessary for maximum production of secondary metabolites. In the present study, screening of important nutrient constituents and process parameters were carried out for the production of red pigment by *M. purpureus* MTCC 369 under submerged fermentation.

EFFECT OF NITROGEN SOURCES ON BIOPIGMENT PRODUCTION

The effect of different nitrogen sources on pigment production was investigated and it was observed that these have significant effect on quality and quantity of pigment produced by *M. purpureus* MTCC 369. The various nitrogen sources such as pea pod, taro leaves, green gram waste, soya okra and peptone were supplemented individually added in media. Though the peptone showed the higher absorbance but keeping in view the economics and availability of sources, agro-industrial by-products can be preferred. Among all the other nitrogen sources tested, pea pod gave highest pigment production (Fig. 1). It was also observed that pigment production

obtained from pea pod (6.93 AU/g) was comparable with the peptone (7.22 AU/g). The low production was obtained in case of green gram waste (5.97 AU/g). It has been also observed that the red pigment dominated when a medium was supplemented with pea pod and kinnow peel powder. The supplementation of external nitrogenous compounds in fermentation media showed a positive impact on water soluble pigment production. Though the literature on the use of above tested nitrogen sources for microbial pigment production is very scarce, yet it has been reported that organic nitrogen sources gave better yield than inorganic nitrogen sources by *Monascus* sp. (Pastrana *et.al.,* 1995; Dufosse *et.al.,* 2005). It has been also observed that the pigment production varies with nitrogen supplementation for pigment production by *Monascus* sp. (Jung *et.al.,* 2003).

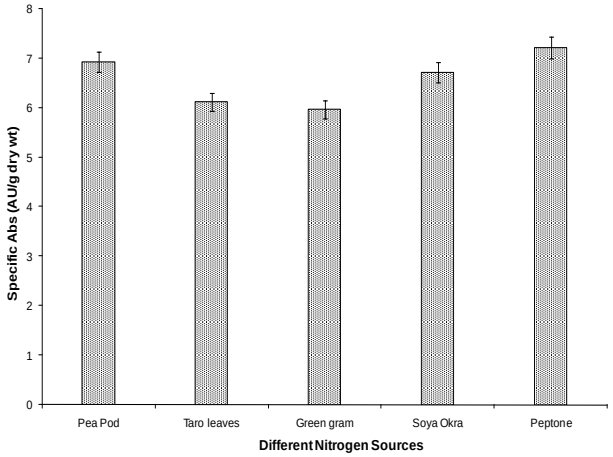

Fig. 1. Effect of different nitrogen sources on pigment production using *M. purpureus* MTCC 369

EFFECT OF TRACE METALS ON BIOPIGMENT PRODUCTION

The supplementation of fermentation media with trace elements (zinc sulfate, ferrous sulfate, manganese sulphate heptahydrate, magnesium sulfate, calcium chloride) was carried out individually to investigate the effect of each metal ion on pigment production (Fig. 2). Magnesium sulphate heptahydrate showed the stimulatory effect on pigment production, whereas ferrous sulfate, calcium chloride showed less mycelia growth. Some stimulatory effect has also been attributed by magnesium sulfate heptahydrate and zinc sulfate. It has been reported that the trace metals have important effect on secondary metabolism (Weinberg, 1989). Our results were found to be comparable with previous studies in which magnesium sulfate heptahydrate and manganese sulfate had contributed significant effect, while calcium chloride and ferrous sulfate showed little impact when added into the medium containing dextrose using *Monascus* sp. MTCC 369 (Ahmad *et al.,* 2009). However, in separate studies by previous workers ferrous sulfate has also showed the stimulatory effect on pigment production using *Monascus purpureus* (Lee *et al.,* 2001). The variations in the results may be due to strain specificity and other components of medium.

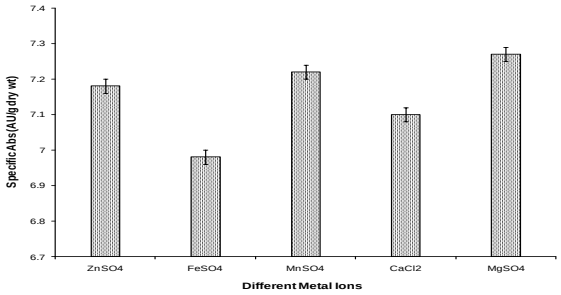

Fig. 2. Effect of different metal ions on pigment production using *M. purpureus* MTCC 369

EFFECT OF PH OF THE FERMENTATION MEDIA ON PIGMENT PRODUCTION

The effect of pH on biopigment production was monitored by varying the pH (5-7.5) of the fermentation media. The results (Fig. 3) indicated maximum pigment production in the pH range of 6.0 to 7.0 and the maximum production of red coloured pigment was observed at pH 6.5 (7.25 AU/g). It has been previously reported that *Monascus* sp. are able to grow at wide range of pH values (2.5-8) with maximum in the range of 4-7 (Carvalho *et al.,* 2005). At low pH, the yellow pigment predominates and at high pH red pigment dominates (Mukherjee and Singh, 2011). The pH range of 5.5-8.5 favoured the red pigment production in Lin media containing rice powder by submerged fermentation using *Monascus purpureus* ATCC 16365 (Lee *et al.,* 2001). Further, low pH conditions (2.0-4.0) favoured yellow pigment production while high pH condition (6.0-8.0) dominated the production of red pigment production in chemically defined media using *Monascus purpureus* NFCCI 1756 (Mukherjee and Singh, 2011).

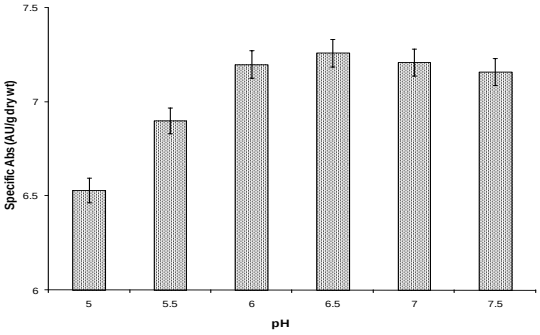

Fig. 3. Effect of pH on pigment production using *M. purpureus* MTCC 369

EFFECT OF TEMPERATURE ON BIOPIGMENT PRODUCTION

The effect of temperature on biopigment production was studied by cultivating *M. purpureus* MTCC 369 in fermentation media with kinnow peel waste and pea pod at temperature range of 25-40°C. Good biopigment production was observed in the temperature range of 30-35°C (Fig. 4). Biopigment production increased with temperature until 35°C and then decreased with further increase in temperature. The results are comparable with previous literature which showed that

incubation of 30-35 °C favoured best growth and pigment production (Lin *et al.*, 2008). The optimal conditions for *Monascus* spp. reported previously are 32 °C at pH 6.0 after incubation of 8 days (Carvalho *et al.*, 2005) in a media containing rice & cassava bagasse. In further experimentation, a temperature of 35°C was selected.

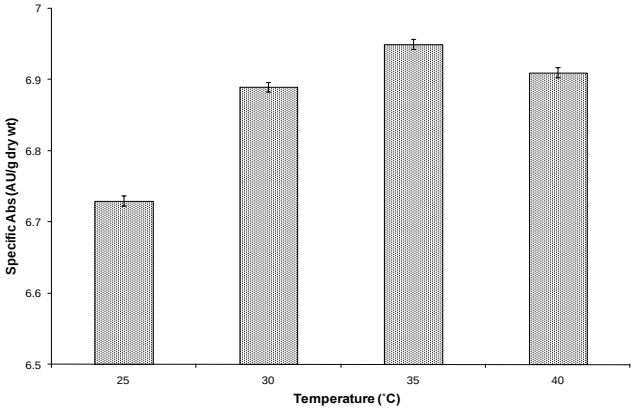

Fig. 4. Effect of temperature on pigment production using *M. purpureus* MTCC 369

EFFECT OF INCUBATION TIME ON BIOPIGMENT PRODUCTION

The effect of incubation period on biopigment production by *M. purpureus* MTCC 369 has been investigated (Fig. 5). The amount of pigment increased as the fermentation time increased. Pigment production was observed on 3rd day after incubation and continued to accumulate throughout the fermentation period. However, maximum pigment production was observed at 9th day (7.26 AU/g) and further increase in incubation period, a decrease in pigment production has been observed. It has been reported in previous literature that the maximum pigment yield was observed on 16th day of inoculation using *Monascus purpureus* MTCC 410 (Dikshit and Tallapragada, 2011) and on 10th day after incubation using *Monascus purpureus* MTCC 1090 and *Monascus purpureus* NFCCI 1756 (Chatterjee et al., 2009; Mukherjee and Singh, 2011). The variations in incubation time to achieve maximum pigment production may be due to strain specificity.

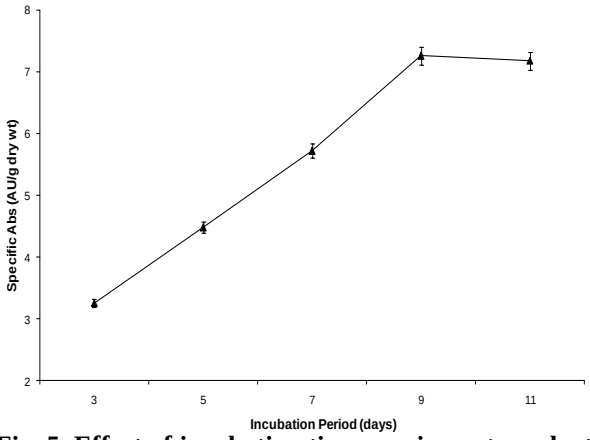

Fig. 5. Effect of incubation time on pigment production using *M. purpureus* MTCC 369

EFFECT OF DIFFERENT SOLVENTS ON PIGMENT EXTRACTION

The pigments were extracted from the fermentation broth using methanol (90%), DMSO (90%), and using different concentrations of ethanol (80%, 90% & 95%). The results indicated that ethanol gave maximum pigment production, whereas methanol was found to be less effective solvent for pigment extraction (Fig. 6). Since ethanol is cheap, volatile and non toxic solvent, it can be good choice for further experiments (Carvalho et al., 2005). Keeping in view the effectiveness of ethanol for pigment extraction, the different concentrations of ethanol have been taken and among them 90% ethanol was found best (7.35 AU/g) followed by 95% ethanol (7.26 AU/g).

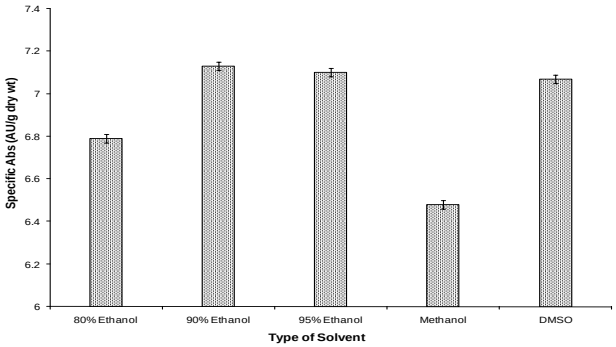

Fig. 6. Effect of different solvents on pigment production using *M. purpureus* MTCC 369

CONCLUSIONS

The obtained results depicted that kinnow peel powder served as a good substrate for the growth of *Monascus purpureus* MTCC 369, which resulted in considerable amount of pigment production. Among all the other nitrogen sources tested, pea pod produced the higher pigment. The stimulatory effect was observed with magnesium sulphate heptahydrate on pigment production whereas ferrous sulphate, calcium chloride showed less mycelia growth. The pigment production was seen after 9 days of incubation at pH 6.5 with a temperature of 35°C in submerged fermentation. However, further studies are required for the complete characterization of new pigment particularly the assessment of its potential in food industry.

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