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OPTIMIZATION OF APPLE-POMACE BASED MEDIUM FOR PIGMENT PRODUCTION BY *MICROCOCCUS FLAVUS*

PARAMITA DEB* AND M. J. MADHUGIRI
Department of Microbiology, Shivaji Science College, Congress Nagar, Nagpur - 440 012, Maharashtra
E-mail: paramitadeb1985@gmail.com

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***Corresponding author**

ABSTRACT
The present studies were undertaken by using apple pomace wastes for pigment production using microbial technology. The substrate used was by-product of apple juice process industry comprising of peel, seed and remaining solid parts. Apple pomace is prepared by mechanically dehydrating apple waste at 60 + 1°C till moisture content reached 4 + 1°C in 3-4h. It is then grind to fine powder. Media originally proposed by Devender Attri and V.K Joshi was modified by incorporating apple pomace. The experiment was further carried out to find various optimized parameters for maximum biomass and pigment production by *Micrococcus flavus* such as pH and temperature. Effect of other factors includes carbon and nitrogen source. Pigment produced was then detected through Paper Chromatography and it was found to be β-carotene when compared with standard results.

INTRODUCTION

The search for novel, effective, safe and affordable bicolor to meet the demands in food industry is one of the most important tasks to pursue production of natural colors for human consumption. Natural colors have been added in food from earlier times, but with increase in demands, synthetic colors like coal tar dyes were developed and dominated the natural colors. The food industry has become increasingly interested in use of microbial technology to produce colors due to the growing public concern over the adverse health effects of synthetic colors (Lin and Demain, 1991). The availability of natural colors developed through biotechnological approaches in the human diet is considered to be most important with respect to health because of their action as provitamins (Olson, 1989; Johnson and Schroeder, 1995), Antioxidants (Burton, 1991) or possible tumor inhibiting agents (Bendich, 1991). Carotenoids are a class of photosynthetic pigments and are of widespread occurrence in nature. Various types of carotenoids are isolated from higher plants like green, brown, red and blue green algae. Besides this Crypto monads and photosynthetic bacteria are also used. Carotenoids are responsible for many of the brilliant red, orange and yellow colors of edible fruits and vegetables. Carotenoids have Vitamin-A activity and are associated with an array of biological functions like antioxidants, anticancer activity, modulation of detoxifying enzyme, enhancing immune system. Due to hazardous nature of synthetic dyes, there is an increased commercial interest in using micro-organisms as a color source. Many plant sources are also useful for bicolor production. A number of micro-organisms (*Rhodotorula, Sarcina, Cryptococcus, Monascus-purpureus, Phaffia rhodozyme, Bacillus, Micrococcus* sp.) produce pigments, but production medium is too complex and requires a number of expensive chemicals (Sandhu and Joshi, 1997). The present study deals with the use of apple waste product known as apple pomace.

MATERIALS AND METHODS

The materials for the present study consist of apple pomace *i.e.* waste material obtained after extracting apple juice. Fresh apple was taken and its juice was extracted. The remaining part known as apple waste was mechanically dehydrated at 60 ± 1°C in a hot air oven till moisture content of dried apple pomace reached 4 ± 1°C in 3-4h. The dried apple pomace was then ground into fine powder using a grinder. The dried powder was sieved through mesh sieve to get desired substrate for pigment production. The powdered form of apple pomace was then used in media preparation. The composition of which is given below:
Apple-pomace based media (g/100mL)

Beef extract -1g; Sodium chloride -0.5g; Peptone -0.5g; Distilled water -100mL; Yeast extract -1.5g; Glucose -0.5g; Apple pomace -4g

Three sets of tubes of above mentioned media was prepared by maintaining pH at 6, 7 and 7.5 respectively. The medium was autoclaved at 121°C for 15 minutes. The cells of *Micrococcus flavus* obtained from National Centre for cell science, Pune were inoculated in the tubes of each set. The inoculated tubes were then incubated at three different temperatures *i.e.* 30°, 35° and 37°C at incubator. The growth of the organism was observed in the form of turbidity in tubes.

The observations were recorded after 24, 48, 72 and 96h. The tube showing maximum turbidity was selected for growth of organism. Optimization of temperature, pH and period of incubation was determined for maximum pigment production by *Micrococcus flavus*.

The other variables which were incorporated in the medium includes carbon and nitrogen source. The carbon source which was tested individually includes glucose, lactose and fructose at 0.5g/100mL each. Likewise nitrogen source such as sodium nitrate, peptone and potassium nitrate were also tested individually at 0.5g/100mL. Based on the appearance of turbidity measured by colorimeter, fructose and sodium nitrate were identified as the best source of carbon and nitrogen. Therefore these two factors were further subjected for refinement of actual concentration at which bacterial growth would be at maximum. Therefore five concentrations *i.e.* 0.2, 0.4, 0.6, 0.8 and 1.0g/100mL were individually tested and most effective concentration was identified.

RESULTS

The experiment was carried out to find various optimized parameters for maximum biomass and pigment production, optimization of physical agents on apple-pomace based media by taking optical density (O.D) in a colorimeter at 540nm. It was observed that pH 6, temperature of 35°C and 4 days period was found to be optimum for biomass production. In order to determine enhanced carotenoid production, carbon source in the form of glucose, fructose and lactose were investigated and were studied for biomass production. By the use of fructose, biomass and pigment production enhanced. Pigment production by *M flavus* along with A-P based media was standardized and observed that fructose when used from 0.2% to 1% concentration, the highest biomass production was observed at 0.6% level followed by 1% fructose concentration. In rest of the fructose concentrations the biomass production was very low (Table 1). It was observed that the biomass production was directly correlated with caretonoid production. From this experiment it can be stated that 0.6 % fructose was found to be optimum for enhanced biomass and pigment production. Similarly, effect of nitrogen source on biomass and pigment production was also investigated by using *M flavus* on A-P based media along with nitrogen sources which include sodium nitrate ($NaNO_3$), potassium nitrate (KNO_3) and peptone. It was observed that sodium nitrate has incrementally enhanced biomass production followed by peptone. However, the medium containing potassium nitrate has yielded less biomass and pigment production. The effect of sodium nitrate at different concentration in relation to biomass and pigment production was determined by using 0.2 to 1.0% concentration. The growth of biomass was observed to be more at 0.6 % followed by 0.4% (Table 2). In order to investigate the type of carotenoid produced by the organism during biomass production, paper chromatography was performed and Rf value was calculated which was 1.2 and correlated with β-carotene. The experiment carried out suggest that *M flavus* on A-P based media produce more pigmentation under the influence of carbon and nitrogen source.

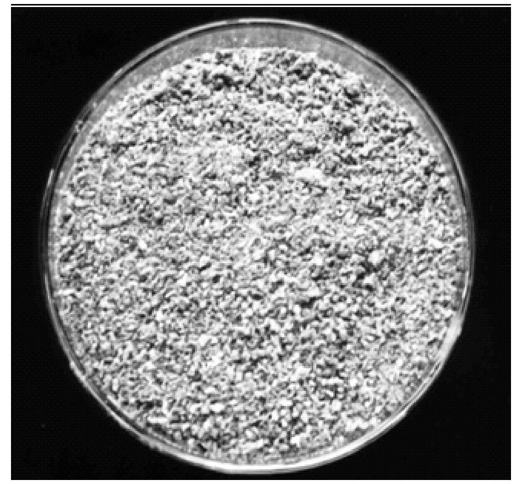

Figure 1: Apple – pomace Used As a Substrate

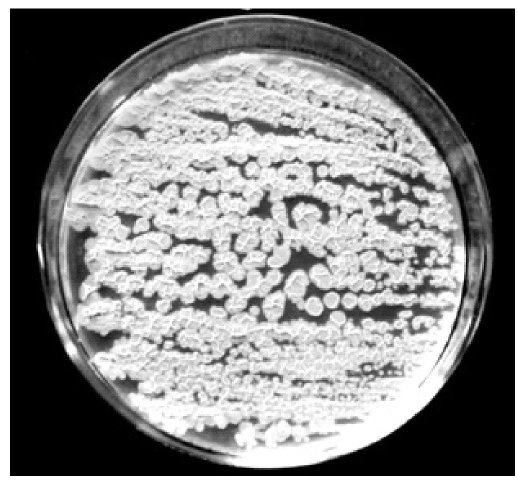

Figure 2: Growth of *Micrococcus flavus* on Apple Pomace Based Medium Showing Pigent Production

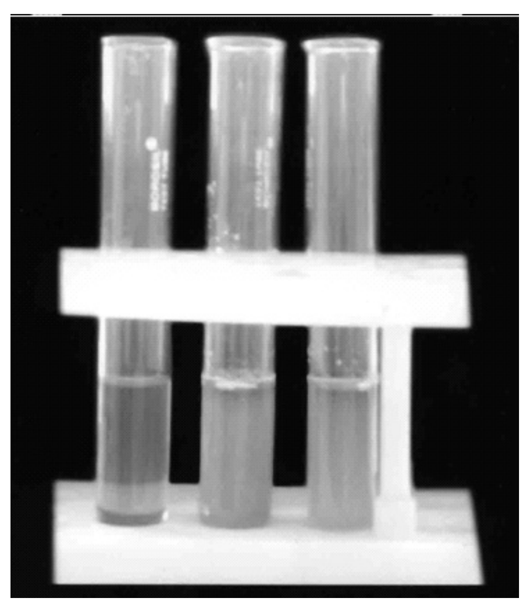

Figure 3: Effect of Different C- Sources [Glucose (1), Fructose (2), Lactose (3)] on Growth of Organism Observed as Turbidity

Table 1: Effect of carbon source and concentration on growth and pigment production by *M flavus* on A-P based media

Carbon source	A	B	(B-A)
Glucose	0.01	0.17	0.16
Fructose	0.04	0.22	0.18
Lactose	0.03	0.20	0.17
Different concentration of Fructose	A	B	(B-A)
0.2	0.01	0.02	0.01
0.4	0.02	0.10	0.08
0.6	0.04	0.21	0.17
0.8	0.11	0.17	0.06
1.0	0.08	0.18	0.10

A = O.D. (withour organism)A; B = O.D.(with organism)B; (B-A) =Growth obtained (B-A)

Table 2: Effect of nitrogen source and concentration on growth and pigment production by *M flavus* on A-P based media

Nitrogen source	A	B	(B-A)
Sodium nitrate	0.42	0.54	0.12
Potassium nitrate	0.01	0.06	0.05
Peptone	0.19	0.29	0.10
Different concentration of Sodium nitrate	A	B	(B-A)
0.2	0.35	0.39	0.04
0.4	0.39	0.46	0.07
0.6	0.43	0.55	0.12
0.8	0.47	0.53	0.06
1.0	0.49	0.51	0.02

A = O.D. (withour organism)A; B = O.D.(with organism)B; (B-A) =Growth obtained (B-A)

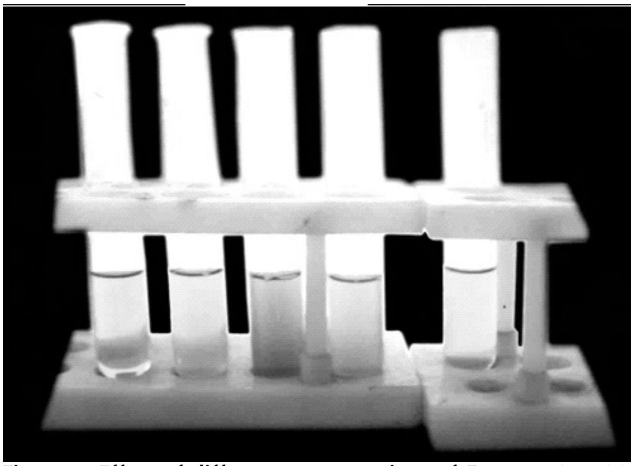

Figure 4: Effect of different concentrations of Fructose (0.2(1), 0.4(2), 0.6(3), 0.8(4), 1.0(5)) on growth of organism as turbidity

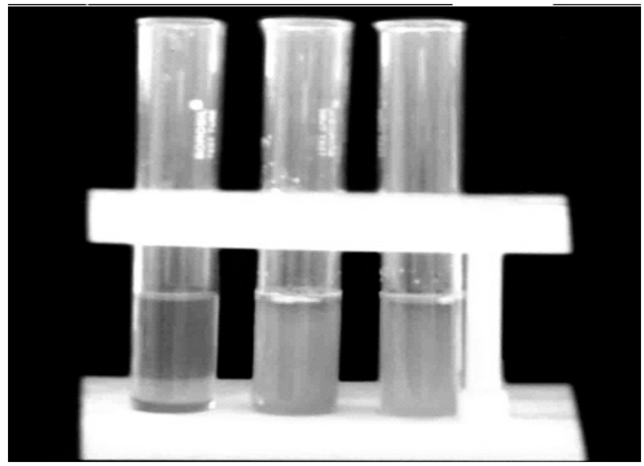

Figure 5: Effect of Different N-Sources [Potassium Nitrate(1), Sodium Nitrate(2), Peptone (3)] on growth of Organism as turbidity

The composition of Optimized Apple-pomace based media (modified) g/100mL

Beef extract - 1g; Sodium chloride - 0.5g; Sodium nitrate - 0.6g; Distilled water -100mL; Yeast extract -1.5g; Fructose - 0.6g; Apple pomace -4g; Agar -2 g; (pH-6)

DISCUSSION

β-carotene also known as pro-vitamin A, occurs naturally as a component of various agricultural products and particularly as a component of green plant. It is synthesized by the plant and is most important pigment for photosynthesis as it translates the light energy absorbed from chlorophyll. When ingested, β-carotene is converted in animal body to vitamin-A. β-carotene is considered as healthy diet in human body because of its action as pro-vitamin, antioxidant or possibly tumor inhibiting agent. It is also used as a source of yellow or orange color in industries related to textiles, plastic, paints, inks and ceramics etc. The main purpose of the present study was β-carotene pigment production from waste sources like apple pomace. In our country recycling of waste substances is considered as a boon to our economy. In the similar way the waste materials obtained from various fruits and vegetables can be exploited as a substrate for pigment production using microbial technology. Joshi *et al.*, 2003 have reported pigment production by *micrococcus* spp by optimization of apple pomace based medium and fermentation condition. They studied the effect of carbon and nitrogen sources on biomass and carotenoid production. It was observed that AP 4g/100mL have shown maximum yield of biomass and carotenoid in the basic medium. Addition of fructose (0.6%) to AP based medium gave the maximum yield of biomass and carotenoids. Among the different nitrogen sources tried, sodium nitrate at 0.6% gave the highest production of biomass. The studies carried out by us indicate that out of three carbon sources tested such as glucose, fructose and lactose; the medium containing fructose yielded highest biomass and pigment production compared to glucose and lactose. Also effect of nitrogen when tested in a range of 0.2 to 1.0%, highest biomass and pigment production was obtained at 0.6% concentration of sodium nitrate. These results indicate that when A-P based media was supplemented with carbon and nitrogen source at appropriate concentration (fructose -0.6%, sodium nitrate -0.6%) help in enhancing pigment and biomass production. Kelly *et al.*, (2003) have reported importance of apple peels. According to them apple peels contain high concentration of phenol substances which may help in prevention of chronic diseases, therefore they have formulated the procedure to produce healthy ingredients used in healthcare and beauty parlor. We have also reported production of high amount of carotenoid using apple pomace based media with *micrococcus flavus*. Joshi (1998) have also emphasized on the production of value added products from apple pomace due to richness in

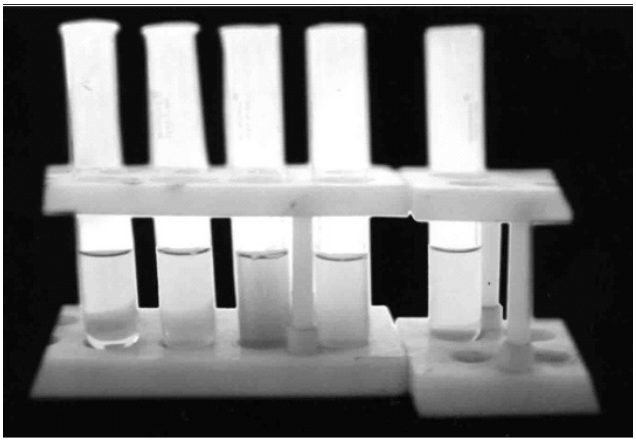

Figure 6: Effect of different concentrations of Sodium Nitrate (0.2(1), 0.4(2), 0.6(3), 0.8(4), 1.0(5)) on growth of organism as turbidity

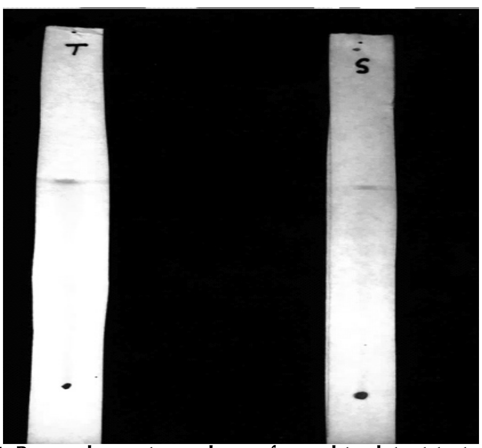

Figure 7: Paper chromatography performed to detect test pigment compared with standard pigment

carbohydrates, dietary fibers and minerals. They have also reported production of different bicolor using *Aspergillus niger*. Recently the food industries has become increasingly interested in using microbial technology to produce natural colors due to the growing public concern over the adverse health effect of synthetic colors (Lin and Demain,1991). Therefore presence of biotechnology based colors in the human diet is being considered healthy because of their action as pro-vitamins (Olson, 1989). Czyzowska *et al*. (2008) carried out preliminary research on utilization of waste byproducts from feed industry. The strain of yeast belonging to fermenting group, fodder, deacidifying and xylose fermenting yeast were cultivated on apple pomace. The product like glycerol followed by succinic acid, lactic acid and acetic acid obtained as main fermentation byproduct.

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