Intl. J. Food. Ferment. Technol. 3(2): 127-134, December, 2013

DOI Number: 10.5958/2277-9396.2014.00340.7

Research Paper

Production and Evaluation of Biocolour (carotenoids) from *Rhodotorula* using Apple Pomace: Effect of Composition of different Nitrogen Sources and Methods of Cell Disruption

V.K. Joshi*, Rakesh Sharma and Aman Girdher

Department of Food Science and Technology, Dr. Y.S Parmar University of Horticulture and Forestry, Nauni, Solan (HP), India

*Corresponding author **:** V.K. Joshi; vkjoshipht@rediffmail.com

Paper no: 70 **Received**: 08 July, 2013 **Received in revised form**: 19 September, 2013 **Accepted**: 9 December, 2013

Abstract

Efforts were made to optimize various parameters for production of carotenoids from *Rhodotorula* isolated from spoiled sauerkraut using apple pomace based media. Among various media tried (with varying concentration of peptone, yeast extract and dextrose), a medium having 40 g/L of apple pomace gave the highest production of carotenoids. Ferrous ammonium sulphate at a concentration of 0.5 % and 0.2 % showed higher carotenoids production when used with apple pomace (10, 20, 30 and 40 g/L) and 60 g/L, respectively. Among all the combinations tried, the highest carotenoid production (46.42 mg/100g) was observed in the medium supplemented with 0.5 % ferrous ammonium sulphate and 40 g/L apple pomace. Whereas, urea at concentrations of 0.1 % and 0.4 % showed higher carotenoids production when used with apple pomace (10, 20, 40 and 60 g/L) and 30 g/L, respectively. Among all the combinations of urea and apple pomace, the highest carotenoid production (31.37 mg/100g) took place in the medium supplemented with 0.1 % urea and 20 g/L apple pomace. Out of ferrous ammonium sulphate and urea, the former proved to be better than later. Use of pestle and mortar with glass beads proved an efficient method for the disruption of *Rhodotorula* cells to extract pigment. Out of various solvents used (acetone, petroleum ether, hexane and ethanol), petroleum ether was the best for the extraction of pigment. The pigment, a carotenoid (with two components) extracted from *Rhodotorula* showed 5 % antioxidant activity**.**

Keywords: Biocolour, carotenoids, *Rhodotorula sp*, apple pomace, nitrogen source.

Colour has always fascinated man and it is the most important characteristics in determining the acceptability of commodities like food. Natural colours are being added in food from the ancient times, but with increase in demand, synthetic colours like coal tar dyes were developed that dominated the natural colours (Pattnaik *et al*., 1999; Sharma and Guleria, 2005; Chattopadhyay *et al*., 2008; Joshi *et al*., 2012). The food industry has become increasingly interested in the use of microbial technology to produce colours due to the growing public concern over the adverse health effects of synthetic colours (Lin and Demain, 1991; Bhat and Mathur, 1998). Moreover, among different pigments of natural origin, carotenoids play a fundamental role in human health so their presence in the human diet and their action as pro-vitamin (Johnson and Schroeder, 1996), antioxidant or possible tumor-inhibiting agents has considerable significance. Naturally occurring food colours are chlorophyll, carotene, lycopene,

anthocyanins, flavonoids and anthoxanthins (Mahajan *et al*., 2000; Downham and Collins, 2000). Natural colours are generally extracted from fruits, vegetables, roots and microorganisms and are often called "*Biocolours*" because of their biological origin (Pattnaik *et al*., 1999; Joshi *et al*., 2003; Joshi and Attri, 2006).

A number of microorganisms (*Rhodotorula, Sarcina, Cryptococcus*, *Monascus purpureus, Phaffia rhodozyma, Chromobacter, Yarrowia* and *Bacillus* sp.) produce pigments, but the production medium is too complex and requires a number of expensive chemicals (Sandhu and Joshi, 1997). There is growing interest in microbial pigments due to their natural character, medicinal properties and nutritive value; production being independent of season, geographical conditions, controllable and predictable yield and safety to use (Francis, 2000; Johnson and Schroeder, 1996). Further, apple pomace (20-30% of crop), a by-product of apple juice processing industry comprising of peel, seed and remaining solid parts is a rich source of carbohydrates, dietary fibers, minerals and vitamin C (Attri and Joshi, 2005) and has potential to support the growth of microorganisms. In the medium for the growth of microorganisms, nitrogen source plays a significant role in addition to other nutrients. The carotenoid pigments of yeasts accumulate in liquid droplet and can thus, be extracted with a variety of organic solvents but efficient extraction from microbial cell is difficult since there are no standard techniques that guarantee the extraction with good yield. The extraction of carotene from *Rhodotorula* sp. requires efficient disruption of the yeast membrane. Therefore, efforts were made in the present study to optimize various parameters such as nitrogen source, extraction method, extraction solvent for the production of carotenoid from *Rhodotorula* sp. using apple pomace based media and the results are described in this communication.

Materials and Methods

Isolation of microorganism: The colour producing microorganisms were isolated from different sources like sauerkraut, soil, fruits (guava, plum) and vegetables using serial dilution technique and directly plated on yeast malt agar and total plate count agar medium using pour plate method (Harrigan amd Mc Cannce, 1966). The plates were incubated at 30°C and 37°C for 3 to 4 days. The coloured colonies from the plate were picked up and then grown on nutrient agar at 30°C for 48-72 hours.

Characterization and identification of the isolated microorganism: Initial identification was made based on the morphological characteristics of the organism isolated from the spoiled sauerkraut sample. These include colony character, pigmentation and observations under the microscope.

Screening of media for growth

Medium Preparation: The ground apple pomace was used to supplement the basic media in which the concentration of apple pomace was gradually increased and the quantity of other ingredients viz, yeast extract, peptone and dextrose were decreased, accordingly (Table 1). Further, ferrous ammonium sulphate and urea at varying concentrations from 0.1 to 0.5 per cent were added in respective media to determine their effect on colour production. Each medium was prepared as per the standard method, poured in petriplates and then, after solidification inoculation of 80 µl cells were made on the solid media and spread by glass spreader and kept in an incubator at 25-30°C. The pH of the medium was kept constant. The area covered, yield of biomass and carotenoids were recorded in each plate.

Disruption of Rhodotorula sp. cells by physical methods: The cells of *Rhodotorula* sp were disrupted by different physical methods (pestle and mortar with or without glass beads). After disruption and extraction, the extract was analyzed for carotenoids as per the standard method (Ranganna, 1997). The carotene contents from disintegrated cells were extracted by using different organic solvents such as acetone, petroleum ether, ethanol and hexane, separately (Valduga *et al*., 2009).

Analyses

Characterization: A suitable quantity of dried cells after harvest from the surface of media was scrapped and extracted in appropriate quantity (30 ml) of solvent. The solvent selected for the study were acetone, petroleum ether, hexane and ethanol. The clear supernatant was used for estimation of maximum absorption at different wavelengths under UV-Vis spectrophotometer (Shimadzu-30 make) both for broad (200-1100) and narrow range (400-600) at 20 nm intervals. At particular wavelength, the graph represented some peaks that showed the presence of a compound in the sample.

Quantification of the pigment extracted: Method used for the quantification of carotenoids was followed as given by Ranganna, (1997).

Table 1: Composition of apple pomace (10-60g) based medium with different concentration of ferrous ammonium sulphate

Constituents (g/L)	**Apple pomace based media**				
Apple pomace based media (10g)					
	T_1	T_2	T_3	T_4	T_5
Peptone	2.5	2.5	2.5	2.5	2.5
Yeast extract	1.25	1.25	1.25	1.25	1.25
Glucose	1	1	1	1	1
FAS	1	2	3	4	5
Distilled water	1	1	1	1	1
pH	7±0.2	7±0.2	7±0.2	7±0.2	7±0.2
Apple pomace based media (20g)					
	T_6	T_7	T_8	T_9	T_{10}
Peptone	2.5	2.5	2.5	2.5	2.5
Yeast extract	1.25	1.25	1.25	1.25	1.25
Glucose	0.5	0.5	0.5	0.5	0.5
FAS	1	2	3	4	5
Distilled water	1	1	1	1	1
pH	7±0.2	7±0.2	7±0.2	7±0.2	7±0.2
Apple pomace based media (30g)					
	T_{11}	T_{12}	T_{13}	T_{14}	T_{15}
Peptone	2.5	2.5	2.5	2.5	2.5
Yeast extract	1.25	1.25	1.25	1.25	1.25
Glucose	0.5	0.5	0.5	0.5	0.5
FAS	1	2	3	4	5
Distilled water	1	1	1	1	1
pH	7±0.2	7±0.2	7±0.2	7±0.2	7±0.2
Apple pomace based media (40g)					
	T_{16}	T_{17}	T_{18}	T_{19}	T_{20}
Peptone	1.25	1.25	1.25	1.25	1.25
Yeast extract	0.65	0.65	0.65	0.65	0.65
Glucose	0.5	0.5	0.5	0.5	0.5
FAS	1	2	3	4	5
Distilled water	1	1	1	1	1
pH	7±0.2	7±0.2	7±0.2	7±0.2	7±0.2
Apple pomace based media (60g)					
	T_{21}	T_{22}	T_{23}	T_{24}	T_{25}
Peptone	1.25	1.25	1.25	1.25	1.25
Yeast extract	0.65	0.65	0.65	0.65	0.65
Glucose	0.5	0.5	0.5	0.5	0.5
FAS	1	2	3	4	5
Distilled water	1	1	1	1	1
pH	7±0.2	7±0.2	7±0.2	7±0.2	7±0.2

FAS= Ferrous Ammonium Sulphate

Table 2: Composition of apple pomace (10-60g) based medium with different concentration of urea

Constituents (g/L)	Apple pomace based media				
Apple Pomace based media (10g)					
	T_1	T_2	T_3	T_4	T_5
Peptone	2.5	2.5	2.5	2.5	2.5
Yeast extract	1.25	1.25	1.25	1.25	1.25
Glucose	1	1	1	1	1
Urea	1	2	3	4	5
Distilled water	1	1	1	1	1
pH	7±0.2	7±0.2	7±0.2	7±0.2	7±0.2
Apple pomace based media (20g)					
Peptone	2.5	2.5	2.5	2.5	2.5
Yeast extract	1.25	1.25	1.25	1.25	1.25
Glucose	0.5	0.5	0.5	0.5	0.5
Urea	1	2	3	4	5
Distilled water	1	1	1	1	1
pH	7±0.2	7±0.2	7±0.2	7±0.2	7±0.2
Apple pomace based media (30g)					
Peptone	2.5	2.5	2.5	2.5	2.5
Yeast extract	1.25	1.25	1.25	1.25	1.25
Glucose	0.5	0.5	0.5	0.5	0.5
Urea	1	2	3	4	5
Distilled water	1	1	1	1	1
pH	7±0.2	7±0.2	7±0.2	7±0.2	7±0.2
Apple pomace based media (40g)					
Peptone	1.25	1.25	1.25	1.25	1.25
Yeast extract	0.65	0.65	0.65	0.65	0.65
Glucose	0.5	0.5	0.5	0.5	0.5
Urea	1	2	3	4	5
Distilled water	1	1	1	1	1
pH	7±0.2	7±0.2	7±0.2	7±0.2	7±0.2
Apple pomace based media (60g)					
Peptone	1.25	1.25	1.25	1.25	1.25
Yeast extract	0.65	0.65	0.65	0.65	0.65
Glucose	0.5	0.5	0.5	0.5	0.5
Urea	1	2	3	4	5
Distilled water	1	1	1	1	1
pH	7±0.2	7±0.2	7±0.2	7±0.2	7±0.2

FAS= Ferrous Ammonium Sulphate

Antioxidant Activity of Pigment Produced: Antioxidant activity of the pigment produced by *Rhodotorula* sp.was measured by using DPPH (2, 2- diphenyl-1-picrylhydrazyl) solution method using methanol as a blank according to the standard method (Brand-Williams *et al*., 1995)

Results and Discussion

Isolation of coloured microorganisms and their characterization: Colour producing microorganisms were isolated from various sources. Out of two isolates, a pink colour producing microorganism was isolated from the sauerkraut sample. On the basis of colour and appearance, it was identified as *yeast*. The microscopic examination showed the cells were ovoidal and a few more elongated and were identified as *Rhodotorula* (Fig 1).

Screening of apple pomace based media

Effect of apple pomace: Significant differences in the area covered, yield of biomass and carotenoid production in all

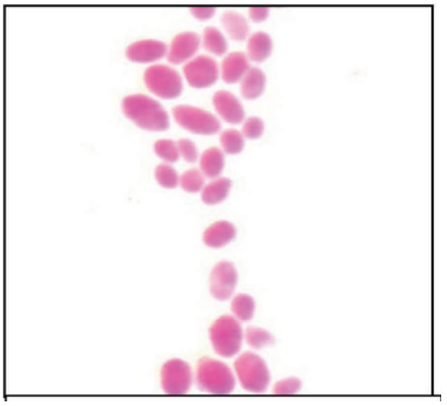

Fig. 1: Photomicrograph of *Rhodotorula sp.*

the treatments were observed (Fig 2). By increasing the concentration of apple pomace from 10 g/L (M_1) to 60 g/ (M_5), the area covered by microbe starts decreasing and the maximum area covered was found in medium M_1 having 10g/L of apple pomace. It was observed that reduction in the ingredients like peptone and yeast extract by 1/4th and increase in apple pomace up to 40 g/L has increased the pigment production (48.4 mg/100g). Further, the medium supplemented with higher concentration of apple pomace (40 g/L) resulted into high pigment production. Similar results have been reported by Joshi and Attri (2006) by using 50 g/L apple pomace in the medium. Thus, apple pomace must have supplied most of the essential nutrients required for the pigment production by *Rhodotorula*.

Effect of nitrogen sources: Various nitrogen sources used under study showed significant differences on the production of carotenoids. The highest production of

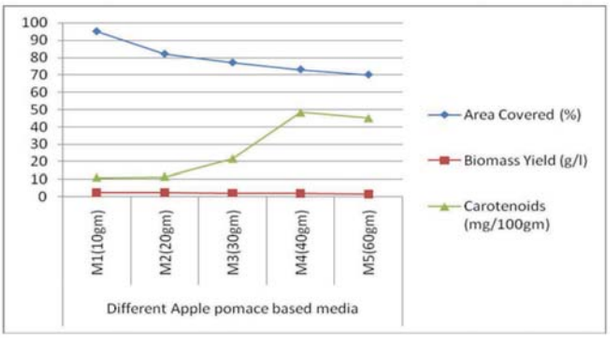

Fig. 2: Optimization of apple pomace based medium for the pigment production by *Rhodotorula* sp.

pigment was recorded in ferrous ammonium sulphate (46.42 mg/100g carotenoid). This is in line with the results reported earlier (Sandhu and Joshi, 1997; Joshi and Attri, 2006). Urea was not found to be much efficient nitrogen source for pigment production by *Rhodotorula* because the colour produced by *Rhodotorula* with urea as a nitrogen source was less bright than with ferrous ammonium sulphate.

Effect of varying concentration of ferrous ammonium sulphate: By increasing the concentration of ferrous ammonium sulphate (0.1% to 0.5%), irrespective of concentration of apple pomace, the per cent area covered decreased, but the carotenoid production showed an increasing trend (Table 3). Thus, ferrous ammonium sulphate might have stimulated the *Rhodotorula* yeast cells to produce much pigment at its higher concentration. However, increasing apple pomace concentration beyond 20g/l, the area covered by the microbe and the biomass yield decreased, but carotenoid content increased. It was found that at 40g/L apple pomace concentration, the carotenoid production was increased from 19.33 mg/100g to 46.42 mg/100g with increase in nitrogen source (0-0.5%). The ferrous ammonium sulphate enhanced the *Rhodotorula* yeast cells to produce more quantity of pigment at its higher concentration due to making available iron, sulphur besides nitrogen. However, increasing the concentration of apple pomace beyond 40g/L, the carotenoid production decreased.

Effect of varying concentration of urea: It was observed that by varying the concentration of urea from 0.1 - 0.5 per cent, carotenoid production decreased, irrespective of concentration of apple pomace in the medium. Thus, urea does not seem to have much role in pigment production by *Rhodotorula* (Table 4). It is clear from the data that at 0.2 % concentration of urea with 20 g/L apple pomace, biomass yield was found to be the maximum and carotenoids were observed maximum with 0.1% urea. Thus, at higher concentration urea may have toxic effect rather than stimulatory factor for pigment production.

Optimization of method for disruption of cells: Different methods were attempted for the disruption of *Rhodotorula sp*. cell (Table 5) and the results showed that maximum carotenoid production (57.46 mg/100g of dry cell weight basis) was obtained by grinding the dry cell powder (from FAS as nitrogen source) with glass beads in pestle and mortar. Similarly, maximum carotenoid production (16.13 mg/100g of dry cell weight basis) was

Table 3: Effect of varying concentration of ferrous ammonium sulphate in apple pomace (10-60g) based media on different parameters

Parameters	Treatments					
	Control(0.0)	FAS (0.1)	FAS (0.2)	FAS (0.3)	FAS (0.4)	FAS (0.5)
Apple pomace (10 g/L)						
Area covered (%)	95	90	87	85	84	80
Biomass Yield (g/L)	1.0	1.0	1.0	1.0	0.7	1.0
Carotenoids (mg/100g)	11.01	11.6	12.26	13.6	24.47	26.26
Apple pomace (20 g/L)						
Area covered (%)	82	98	87	95	88	85
Biomass Yield (g/l)	1.2	1.6	1.2	1.1	1.1	1.1
Carotenoids (mg/100g)	14	13.56	13.41	32.39	21.95	41.55
Apple pomace (30 g/L)						
Area covered (%)	73	98	93	92	90	84
Biomass Yield (g/L)	1.1	1.3	1.5	1.6	1.3	1.3
Carotenoids (mg/100g)	14	25.74	26.13	24.19	26.15	27.33
Apple pomace (40 g/L)						
Area covered (%)	70	93	90	85	88	83
Biomass Yield (g/L)	1.0	1.2	1.0	1.1	1.4	1.1
Carotenoids (mg/100g)	19.33	45.63	34.66	36.72	24.14	46.42
Apple pomace (60 g/L)						
Area covered (%)	75	90	83	84	87	81
Biomass Yield (g/L)	1.1	1.3	1.1	1.1	1.3	1.2
Carotenoids (mg/100g)	19.33	31.12	42.40	37.45	31.60	31.26

FAS= Ferrous Ammonium Sulphate

Table 4: Effect of varying concentration of urea with apple pomace (10-60g) based media on different parameters

Parameters	Treatments					
	Control (0.0)	Urea (0.1)	Urea (0.2)	Urea (0.3)	Urea (0.4)	Urea (0.5)
Apple pomace (10 g/L)						
Area covered (%)	75	80	95	93	88	85
Biomass Yield (g/L)	1.0	0.5	0.6	0.6	0.5	0.5
Carotenoids (mg/100g)	19.33	25	24.66	21.22	23.86	22
Apple pomace (20 g/L)						
Area covered (%)	82	90	93	96	85	82
Biomass Yield (g/L)	1.2	1.1	1.5	1.0	0.9	0.9
Carotenoids (mg/100g)	13.33	31.37	11.15	24.26	20.14	21.11
Apple pomace (30 g/L)						
Area covered (%)	73	98	94	85	90	84
Biomass Yield (g/L)	1.1	1.5	1.3	1.4	1.0	0.7
Carotenoids (mg/100g)	21.66	11.20	11.38	10.42	11.93	10.86
Apple pomace (40 g/L)						
Area covered (%)	87	85	80	88	85	82
Biomass Yield (g/L)	1.1	1.2	1.0	1.3	1.1	1.0
Carotenoids (mg/100g)	17.57	15.27	15.06	11.23	11.45	11.93
Apple pomace (60 g/L)						
Area covered (%)	75	95	92	87	88	84
Biomass Yield (g/L)	1.6	1.3	1.5	1.0	1.0	1.1
Carotenoids (mg/100g)	19.33	17.38	10.75	15.26	14.60	11.69

obtained by grinding the dry cell powder (from urea as nitrogen source) with glass beads in pestle and mortar.

Table 5: Effect of different extraction method on the pigment extraction from *Rhodotorula* cells

Method	Carotenoids (mg/100g)
Pestle and mortar (FAS)	51.0
Pestle and mortar with glass beads (FAS)	57.46
Pestle and mortar (Urea)	16.13
Pestle and mortar with glass beads (Urea)	18

Effect of different solvents on the pigment extraction: Various organic solvents were tested to extract the carotene from disrupted cells of *Rhodotorula* sp (Fig 3). Although all the solvents tested were able to extract the carotenoids up to some extent, variability in the recovery of pigment was also observed. Maximum carotene (16.8 mg/100g on dry cell weight basis) was obtained by treating the disrupted cells of *Rhodotorula* sp with petroleum ether (30 ml in 1 g of dried biomass).

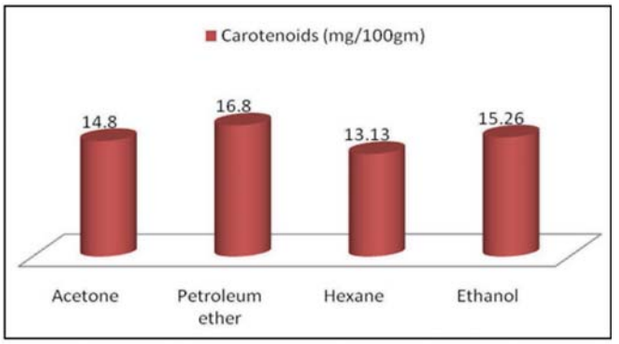

Fig. 3: Effect of different solvents on pigment extraction from *Rhodotorula* cells

Characterization of pigment and its antioxidant activity: It is apparent that the absorbance took place between the range of 400-600 nm. The spectrum of the different samples showed different peaks at different wavelength that indicated that the pigment is not a single compound but combination of more than one compound. The graph confirms the presence of carotenoid compounds because carotenoids have highest absorption in the range of 400-600 mm at 452 nm (Fig. 4). Further, the pigment showed an antioxidant activity of 5 % (DPPH free radical scavenging activity).

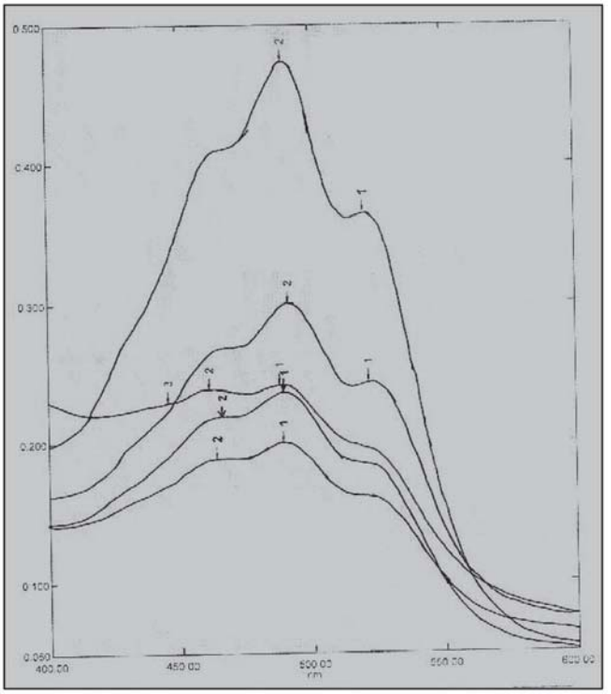

Fig. 4: Graphical representation of the absorbance at different wavelength by the pigment of *Rhodotorula*

Conclusion

It can be conluded that apple pomace can be incorporated as one of the ingredient for the production of pigment from *Rhodotorula* sp. The highest amount of pigment was produced at a level of 40 g/l of apple pomace. Pestle and mortar with glass beads proved to be a better method for extraction of pigment and petroleum ether gave the maximum extraction. Characterization of pigment confirmed it as carotenoid and the pigment had shown antioxidant activity.

References

Attri D and Joshi VK 2005. Optimization of apple pomace based medium and fermentation conditions for pigment production by Micrococcus spp. *Journal of Science and Industrial Research* 598-601.

Bhat RV and Mathur P 1998. Changing scenario of food colours in India. *Current Science* **74**: 198-200.

Brand-Willium W, Cuvelier ME and Berset C 1995. Use of a free method to evaluate antioxidant activity. *Leben Wiss U Tech* **28**: 25-30

Chattopadhyay P, Chatterjee S and Sen S K. 2008. Biotechnological potential of natural food grade biocolorants. *African Journal of Biotechnology* **7** (17): 2972-2985.

Downham A and Collins P 2000. Colouring our foods in the last and

next millennium. *Interl Journal of Food Science and Technology* **35**: 5-22.

Dufosse L 2006. Microbial production of food grade pigments. *Food technology and Biotechnol*ogy **44**:313-321.

Francis FJ 2000. Carotenoids as Food Colourants. *Cereal Food World* **45:**198-203.

Harrigan WF and Mc Cannce E.M. 1966. *Laboratory methods in microbiology*, 2nd Edn. Academic Press, London.

Johnson EA and W Schroeder 1996. Microbial carotenoids.Advances in *Biochemical Enging Biotechnol*ogy **53**: 119-178.

Joshi VK and Attri D 2006. Optimization of apple pomace based medium and fermentation conditions for pigment production by *Rhodotorula* sp. *Proceeding of National Academic Science of India* 76: 171-177

Joshi VK, Attri D, Bala A and Bhushan S 2003. Microbial pigments, *Indian Journal of Bioechnol*ogy **2:** 362-369.

Joshi V.K., Devi M P, Attri D and Sharma Rakesh 2012. Biocolour: Chemistry, Production, Safety and Market Potential. In: Food Biotechnology-Principles and applications. IK Publishers, New Delhi, pp 640-689.

Lin TF and Demain AL 1991. Effect of nutrition of Monascus species on formation of red pigment, *Applied Microbiology and Biotechnol*ogy **36** : 70-75.

Mahajan R, Shukla FC and Shoree M 2000. Commercial use of natural food colours-A review. *Beverage Food World* **27**(1): 44-51.

Masoodi FA, Bakshi P and Shah TA 2005. Bio-colourants: future food ingredients. *Beverage Food World* **32**:27-28.

Pattnaik P, Roy U and Jain P 1999. New Generation additives for food. *Beverage and Food World* **26**(3):36-40

Ranganna S 1997. Handboor of analysis and quality control for fruits and vegetable products. 2nd ed., *Tata Mac Graw Hill Publication Co*., New Delhi. p.112

Sandhu D K and Joshi V K 1997. Development of apple pomace based medium, optimizing pigment production by Rhodotorula and its characterization, *Advance Food Sci*ence **19**: 31-34.

Sharma R and Guleria SPS 2005. Plant Origin Colours- Colours of The Next Millenium. *Young Indian* 19-25: 3-5.

Valduga E, Valerio A, Tatsch PO, Treichel H, Furigo A and Di Luccio M 2009. Assessment of cell disruption and carotenoids extraction from *Sporidiobolus salmonicolor* (CBS 2636). *Bioprocess. Technology* **2**: 234-238.