

Strong Antioxidant Property of Bacterial Canthaxanthin Obtained By Raw Coconut Water Supplementation as an Additional Nutrient Source

Surojit Bera¹, Vamsi Bharadwaj SV¹, Surabhi Chaudhuri¹ and Debjani Dutta^{1*}

¹Department of Biotechnology, National Institute of Technology, M. G. Avenue, Durgapur-713209, West Bengal, India. *Corresponding author: Debjani Dutta, E-mail: debs_2000in@yahoo.com; debjani.dutta@bt.nitdgp.ac.in Ph.: +91-343-275-4034; Fax: +91 343 2547375

Abstract: Microbial carotenoids are gaining industrial importance due to its attractive colour and strong antioxidant activity. Highest canthaxanthin yield till date was obtained by *Dietzia maris* NIT-D with three (03) different aged raw coconut water (tender, moderate and mature) supplementation. Maximum pigment yield was found to be 527.09mg/L in moderately aged coconut water (6-8 months) supplementation. Specific growth rate obtained was 0.0236 h⁻¹ and it increased 1.43 fold from the customised medium of our previous report. The biomass was found to be 18.85g/L and 84.24% reducing sugar was utilised while extracellular protein content increased up to 8 folds by the test bacterium. Radical scavenging activities of the obtained pigment were investigated in terms of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and nitrate scavenging assay. IC₅₀ values from such experiments indicate the efficacy of the extracted pigment in terms of its bioactive potentiality. These findings have robust significance in industry and have applications in health and nutraceuticals owing to its strong antioxidant property.

Keywords: Coconut water, Canthaxanthin, Antioxidant activity, IC₅₀ value, nutraceuticals aspect

1. Introduction

Carotenoids are naturally occurring pigments (chemically hydrocarbon derivatives by nature) synthesised by wide range of microorganisms [9]. Canthaxanthin is a reddish orange keto carotenoid produced by different microbes as a secondary metabolite. It is getting prominence in poultry industry, aqua farming, cosmetics and nutraceuticals industries due to attractive colour and strong antioxidant property [7, 16].

Highest canthaxanthin producing strain till date *Dietzia maris* NIT-D was isolated in our laboratory [9], the yield being 121 mg/L [9]. We are trying to improve the canthaxanthin yield by using different optimisation techniques and media supplement. It was observed that small quantity of moderately aged raw coconut water enhanced the canthaxanthin production significantly along with its normal customised growth medium. In 2004, Domínguez-Bocanegra and Torres-Muñoz [5] reported the use of raw coconut milk as a sole carbon source for overproduction of astaxanthin (an industrially important red-orange carotenoid) by yeast. Coconut (*Cocos nucifera* L.) is a significant member of the family *Arecaceae* and is a highly produced crop in India (119.3 × 10⁵ tons in 2013) [6], as well as all over the world. Coconut water which originates in the fruit after one and half month is a highly nourishing liquid containing enormous range of chemical components like different sugars, sugar alcohols, inorganic ions, vitamins, lipids, amino acids, nitrogenous compounds, organic acids, enzymes, phytohormones *etc.* [2, 3, 20]. In addition to its health benefits, it is a traditional growth supplement for plant tissue culture which reflects its bioactivity along with prominence towards the industry, biotechnology and biomedical field [20].

Till now there has been no report for production (or overproduction) of canthaxanthin with the help of coconut water. The aim of the work was to overproduce canthaxanthin by *Dietzia maris* NIT-D in a new

supplement. Additionally our objective encompassed to study the antioxidant property in terms of different radical scavenging system for future nutraceuticals aspect.

2. Materials and Methods

2.1. Microorganism

The experimental strain *Dietzia maris* NIT-D (accession number: HM151403) was previously isolated and established as an efficient canthaxanthin producer by our laboratory group member. The strain was maintained weekly by routine transfer into brain heart infusion agar (Hi-Media, India) slants and stored at 4°C after being incubated at 25°C for 5 days [9].

2.2. Collection of coconut water

Fresh coconuts of three different categories i.e. tender (3-4 months), moderately mature (6-8 months), very mature (10-12 months) were collected from local cultivators. Coconut water was taken and filter sterilized [5] twice (by 0.22µ autoclaved filter paper, Millipore, USA) and stored in sterile containers at 4°C for further experiments.

2.3. Coconut water supplemented media preparation

In each 250mL Erlenmeyer flasks, coconut water supplemented (40% v/v) customized media (total volume 50 mL in each flask) was prepared with different components of the main growth medium as shown in Table1. Individual medium components were substituted one variable at a time approach with three different varieties of coconut water.

2.4. Inoculum preparation

The cell inoculum was prepared according to Goswami et al. [9].

2.5. Production, extraction and analysis of pigment

Optimized medium was used for large scale pigment production at initial broth pH-5.5, shaker speed of 120 rpm and 25°C. Pigment extraction and analysis was done according to Goswami et al. [9]. Extracted pigment was concentrated using rotary evaporator (Yamato, model RE301) followed by lyophilisation (Digitech Instruments, India). Dried canthaxanthin was stored at 4°C for further experiments. Comparison was done with pure standards of canthaxanthin (Sigma-Aldrich, USA).

2.6. Microbial growth and dry biomass estimation

This process was done according to Domínguez-Bocanegra and Torres-Muñoz [5], with modifications. Cell growth was initially investigated based on the simple Monod equation [$dX/dt=\mu X$, where μ is the specific growth rate and X is the instantaneous biomass concentration].

2.7. Reducing sugar and protein estimation

Total reducing sugar and total protein content of cell free fermented broth was determined at regular interval (every 24hrs) according to Miller [13], and by Lowry's Method [12] respectively.

2.8. DPPH free-radical scavenging activity

Radical scavenging ability using the DPPH[•] (1, 1-diphenyl-2-picrylhydrazyl) of the extracted carotenoid was determined by the method of Aquino *et al.* [1] with minor modifications. Vit-C was used as a positive control.

2.9. Nitrite scavenging activity (NSAs)

Nitrite scavenging activity of the obtained pigment was estimated by the method of Choi *et al.* [4] with minor modifications. Vit-C was used as a positive control.

3. Results and discussion

3.1. Production and supplementation volume optimization for pigment in coco water supplemented medium and pigment analysis

Table 1 shows different medium composition in different aged coconut water. It was observed that highest pigment production of 487.09 mg/L has obtained in E type medium. The medium was supplemented with moderately aged (6-8 months, C2) coconut water while tender (3-4months, C1) and mature (10-12 months, C3) supplemented medium also provided good yield of 383.52 mg/L and 344.73 mg/L pigment respectively (Figure 1). Surprisingly, medium containing all customised components except glucose (B type, Table 1) showed next highest pigment yield (i.e. 354.58 mg/L, 409.31 mg/L and 323.91 mg/L in C1, C2 and C3 supplemented broth respectively (Figure 1). To the best of our knowledge this is the highest reported yield of canthaxanthin production in a medium without glucose. In all cases moderately aged supplementation showed promising result than the other two. The lowest and negligible production was obtained in medium containing only coconut water of different age (J type, Table 1). The yield was 1.85 mg/L, 3.17 mg/L and 1.32 mg/L in C1, C2 and C3 supplemented broth respectively (Figure 1). A significant observation was that moderately aged coconut water showed high efficacy as an extra supplemented nutritional source for pigment production than tender and mature type.

For analysis and confirmation, the filtered extracted pigment was scanned in the wavelength range of 200-1100 nm using UV-Vis spectrophotometer. Maximum absorbance was observed at 473nm. TLC was carried out and on basis of migration, R_f value of extracted pigment was calculated to be 0.48 which is almost similar with 0.49 of standard *trans*-canthaxanthin. HPLC peaks were monitored at 473 nm. The concentrated hexane extract showed a retention time of 7.964 min. Standard *trans*-canthaxanthin showed a retention time of 7.980 min with a 100% peak area. The percentage area of test pigment was calculated to be almost 100%, thereby signifying the purity and confirming that *trans*-canthaxanthin was the major carotenoid produced by our experimental organism i.e. *Dietzia maris* NIT-D (accession number: HM151403).

Microbial production of canthaxanthin is highly dependent on various physical parameters like temperature, pH, bioreactor design etc. and chemical parameters like carbon source, nitrogen source, extra nutritional factors etc. [8]. Coconut water contains a variety of nutritional factors as well as simple sugars and hence can be used as carbon source supplementation. Domínguez-Bocanegra and Torres-Muñoz [5] successfully used raw coconut milk as sole carbon source for astaxanthin production. Prabakaran *et al.* [17] showed large scale production of bacterial endotoxin using coconut water as cheap growth medium component. Hungund *et al.* [10] studied bacterial cellulose production by coconut water along with other fruit juices as cheap carbon source. But till now no report has been made with the use of coconut water for any kind of carotenoid production. In this study, we have seen our experimental organism could not use coconut water as a sole carbon source, however, the highest production of canthaxanthin (527.09 mg/L) was achieved in moderately aged coconut water (6-8months, C2) supplemented with customised medium. Results of percentage optimization of coconut water supplementation suggest that below and above 20% v/v, the strain was lacking nitrogen source though adequate carbon source content was present. This was confirmed by individual composition effect study of the medium (Figure 1). Canthaxanthin yield got decreased when peptone or yeast extract was not present in the medium. Glucose may not be a limiting factor in this study. The medium devoid of free glucose also gave much higher canthaxanthin yield (356.74 mg/L). This could be due to high availability of reducing sugar present in coconut water. Moreover wide range of micronutrients present in coconut water like TCA cycle intermediates [14, 15] may have enhanced the growth of *Dietzia maris* in this study thus leading to very high pigment yield as influential factors on pigment production is also related with cellular metabolism [5].

3.2. Growth characteristics with respect to pigment production, reducing sugar consumption, biomass yield and extra cellular protein levels

The initial reducing sugar content in C2 type coconut water was 16.75 g/L and reduced to 2.59 g/L in day 5 along with the subsequent increase in pigment production from 4.55 mg/L to 527.09 mg/L when the organism was inoculated in the coconut water supplemented medium (Figure 2). Similar result were observed in C1 and C3 supplemented medium with the reduction in reducing sugar level from 7.93 g/L to 2.97g/L and 11.34 g/L to 2.81 g/L respectively. Pigment yield for C1 and C3 also revealed similar increasing pattern as C2 but in lesser

quantity (Figure 2) which is 4.48 mg/L to 403.52 mg/L and 4.65 mg/L to 376.52 mg/L respectively (from day one to day five). The biomass production also increased in C2 medium along with pigment yield (Figure 3). Initial biomass was 1.12 g/L on day one which significantly enhanced to 18.85 g/L on day five. The extracellular protein level also increased from an initial 9.85 mg/L to 77.29 mg/L in fifth day (Figure 3).

The growth curve study showed that coconut water supplementation resulted in shortening of lag phase by 3-4 hours and the strain reached stationary phase in about 70-74 hours with respect to regular customised medium [9]. This could be a reason for a high biomass accumulation (18.85 g/L), which is 2.5 fold greater than previous report of canthaxanthin production with *Dietzia maris* [9]. Specific growth rate was calculated to be 0.0164 h⁻¹ in regular growth medium but increased up to 1.43 fold and was found to be 0.0236 h⁻¹ in coconut water supplemented medium.

Reducing sugar is an important factor for bacterial pigment production. Use of different natural sugar sources were reported for canthaxanthin production like fumaric acid-molasses [16], cheese whey [11] etc. Present study showed *Dietzia maris* NIT-D was using the reducing sugar in coconut water from day one to day five resulting in very high biomass, extracellular protein content and enhanced specific growth rate. This suggests that the growing cells were metabolically very active and may be some unspecified nutrient factors induced the bacterial growth leading to a high canthaxanthin accumulation.

3.3. DPPH free-radical scavenging activity

DPPH free-radical scavenging activity was done to study the radical quenching ability of canthaxanthin obtained from three different types of coconut water supplemented medium. Pure standard trans-canthaxanthin and vitamin C were used as positive control. The radical scavenging activity was measured in terms of % inhibition. It was observed that the pigment obtained from C2 supplemented medium had a higher radical scavenging activity than C1 and C3. Canthaxanthin obtained from C2 type had 22.97% and 82.87% inhibition at 25 µg/mL and 400 µg/mL concentration respectively (Figure 4).

Antioxidant efficacy of extracted pigments was evaluated in terms of their IC₅₀ values. Results showed that IC₅₀ values of pigment obtained from C1, C2, and C3 supplemented medium were 259.74 µg/mL, 224.72 µg/mL and 283.93 µg/mL. Pure standard trans-canthaxanthin and vitamin C showed an IC₅₀ values 210.88 µg/mL and 189.18 µg/mL respectively. Results also showed that vitamin-C was the most potent inhibitor for DPPH.

Some recent reports Gharibzahedi et al. and Venugopalan et al. showed similar kind of results for DPPH inhibition by bacterial canthaxanthin [8, 18]. Canthaxanthin obtained from *Dietzia natronolimnaea* HS-1 showed approximate 80% DPPH inhibition at 0.2 g/mL concentration [8]. Hence moderately aged coconut water supplemented medium can be used as a better radical scavenger producer (i.e canthaxanthin).

3.4. Nitrite scavenging activity (NSAs)

Nitrosamine is a product of reaction between nitrite with amines in protein-rich staple vegetables and meat products which can be a significant threat to animal health leading to cancer [4]. Nitrite is widely used in food processing industries as preservative and colouring [8]. Hence NSA determination of the pigment is important in terms of nutraceutical and healthcare issues. Our obtained pigment showed 39.18%, 53.57% and 47.69% inhibition in C1, C2 and C3 category respectively in 500µg/mL concentration. The results also revealed that Vit-C is (80.92%) more effective (Figure5) in terms of NSAs % rather than pure standard canthaxanthin. This phenomenon indicates C2 type extracted pigment's efficacy as well as clarity in comparison with commercial ones. These results showed a dose-dependent relationship and were quantitatively similar with previous reports of Gharibzahedi et al. [8] and Wang et al. [19]. These studies proved that the extracted pigment may be an alternative source of effective antioxidant in terms of nitrite scavenging activity.

4. Conclusion

From this present study it was observed that moderately aged [6-8 months] coconut water can be used as a cheap nutrient supplement for significant improvement of canthaxanthin yield by *Dietzia maris* NIT-D. Pigment analysis and radical quenching property showed that canthaxanthin obtained by coconut water supplementation could be an efficient radical scavenger. Hence this study will helpful for bacterial pigment production strategies in terms of industrial aspect as well as bioprocess of naturally bioactive molecules.

5. Acknowledgments

The authors express their thanks to “INSPIRE programme” by “Department of Science and Technology (DST)”, Ministry of Science and Technology, New Delhi, Government of India, for fellowship support to Mr. Surojit Bera [IF120552] for carry out this work.

6. References

- [1] R. Aquino, S. Morelli, M.R. Lauro, S. Abdo, A. Saija, and A. Tomaino. (2001). Phenolic constituents and antioxidant activity of an extract of *Anthurium versicolor* leaves. *Journal of Natural Products*. 64(8). pp. 1019-1023.
<http://dx.doi.org/10.1021/np0101245>
- [2] L.R. Brewer, J. Kubola, S. Siriamornpun, T.J. Herald, and Y Shi. (2014). Wheat bran particle size influence on phytochemical extractability and antioxidant properties. *Food Chemistry*. 152. pp. 483-490.
<http://dx.doi.org/10.1016/j.foodchem.2013.11.128>
- [3] S. Chidambaram, C. Singaraja, M.V. Prasanna, M. Ganesan, and M. Sundararajan. (2013). Chemistry of tender coconut water from the cuddalore coastal region in tamil nadu, India. *Natural Resources Research*. 22(2). pp. 91-101.
<http://dx.doi.org/10.1007/s11053-013-9203-y>
- [4] D. Choi, K. Cho, M. Na, H. Choi, Y. Kim, D. Lim, S.J. Cho, and H. Cho. (2008). Effect of bamboo oil on anti-oxidative activity and nitrite scavenging activity. *Journal of Industrial and Engineering Chemistry*. 14. pp. 765-770.
<http://dx.doi.org/10.1016/j.jiec.2008.06.005>
- [5] A.R. Domínguez-Bocanegra, and J.A. Torres-Muñoz. (2004). Astaxanthin hyper production by *Phaffia rhodozyma* (now *Xanthophyllomyces dendrorhous*) with raw coconut milk as sole source of energy. *Applied Microbiology and Biotechnology*. 66. pp. 249-252.
<http://dx.doi.org/10.1007/s00253-004-1686-3>
- [6] FAOSTAT (2014). Food and Agriculture Organization of the United Nations Available: (<http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#anchor>).
- [7] S.M.T. Gharibzahedi, S.H. Razavi, and S.M. Mousavi. (2013). Microbial canthaxanthin: perspectives on biochemistry and biotechnological production. *Engineering in Life Sciences*. 13(4). pp. 408-417.
<http://dx.doi.org/10.1002/elsc.201200153>
- [8] S.M.T. Gharibzahedi, S.H. Razavi, and S.M. Mousavi. (2013). Comparison of antioxidant and free radical scavenging activities of bio colorant synthesized by *Dietzia natronolimnaea* HS-1 cells grown in batch, fed-batch and continuous cultures. *Industrial Crops and Products*. 49. pp.10-16.
<http://dx.doi.org/10.1016/j.indcrop.2013.03.019>
- [9] G. Goswami, S. Chakraborty, S. Chaudhuri, and D. Dutta. (2012). Optimization of process parameters by response surface methodology and kinetic modeling for batch production of canthaxanthin by *Dietzia maris* NIT-D (accession number: HM151403). *Bioprocess and Biosystems Engineering*, 35. pp. 1375-1388.
<http://dx.doi.org/10.1007/s00449-012-0726-0>
- [10] B. Hungund, S. Prabhu, C. Shetty, S. Acharya, V. Prabhu, and S.G. Gupta. (2013). Production of bacterial cellulose from *Gluconacetobacter persimmonis* GH-2 using dual and cheaper carbon sources. *Journal of Microbial & Biochemical Technology*. 5(2). pp. 031-033.
<http://dx.doi.org/10.4172/1948-5948.1000095>
- [11] F. Khodaiyan, S.H. Razavi, and S.M. Mousavi. (2008). Optimization of canthaxanthin production by *Dietzia natronolimnaea* HS-1 from cheese whey using statistical experimental methods. *Biochemical Engineering Journal*. 40. pp. 415-422.
<http://dx.doi.org/10.1016/j.bej.2008.01.016>
- [12] O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall. (1951). Protein measurement with the Folin phenol reagent”, *The Journal of Biological Chemistry*. 193(1). pp. 265-275.
- [13] G.L. Miller. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*. 31. pp. 426-428.
<http://dx.doi.org/10.1021/ac60147a030>
- [14] M.R. Nasri Nasrabadi, and S.H. Razavi. (2010). Use of response surface methodology in a fed-batch process for optimization of tricarboxylic acid cycle intermediates to achieve high levels of canthaxanthin from *Dietzia natronolimnaea* HS-1. *Journal of Bioscience and Bioengineering*. 109. pp. 361-368.
<http://dx.doi.org/10.1016/j.jbiosc.2009.10.013>
- [15] M.R. Nasri Nasrabadi, and S.H. Razavi. (2010). Enhancement of canthaxanthin production from *Dietzia natronolimnaea* HS-1 in a fed-batch process using trace elements and statistical methods. *Brazilian Journal of Chemical Engineering*. 27. pp. 517-529.
<http://dx.doi.org/10.1590/S0104-66322010000400003>

- [16] J.H. Nelis, and A.P. De Leenheer. (1989). Reinvestigation of *Brevibacterium* sp. Strain KY-4313 as a source of canthaxanthin. *Applied and Environmental Microbiology*. 55. pp. 2505-2510.
- [17] G. Prabakaran, S.L. Hoti, A.M. Manonmani, and K. Balaraman. (2008). Coconut water as a cheap source for the production of δ -endotoxin of *Bacillus thuringiensis* var. *israelensis*, a mosquito control agent. *Acta Tropica*. 105. pp. 35-38.
<http://dx.doi.org/10.1016/j.actatropica.2007.09.002>
- [18] V. Venugopalan, S.K. Tripathi, P. Nahar, P.P. Saradhi, R.H. Das, and H.K. Gautam. (2013). Characterization of canthaxanthin isomers isolated from a new soil *Dietzia* sp. and their antioxidant activities. *Journal of Microbiology and Biotechnology*. 23(2). pp. 237-245.
<http://dx.doi.org/10.4014/jmb.1203.03032>
- [19] B. Wang, B. Li, Q. Zeng, and H. Liu. (2008). Antioxidant and free radical scavenging activities of pigments extracted from molasses alcohol wastewater. *Food Chemistry*, 107. pp. 1198-1204.
<http://dx.doi.org/10.1016/j.foodchem.2007.09.049>
- [20] J.W.H. Yong, L. Ge, Y.F. Ng, and S.N. Tan. (2009). The chemical composition and biological properties of coconut (*Cocos nucifera* L.) water. *Molecules*. 14. pp. 5144-5164.
<http://dx.doi.org/10.3390/molecules14125144>

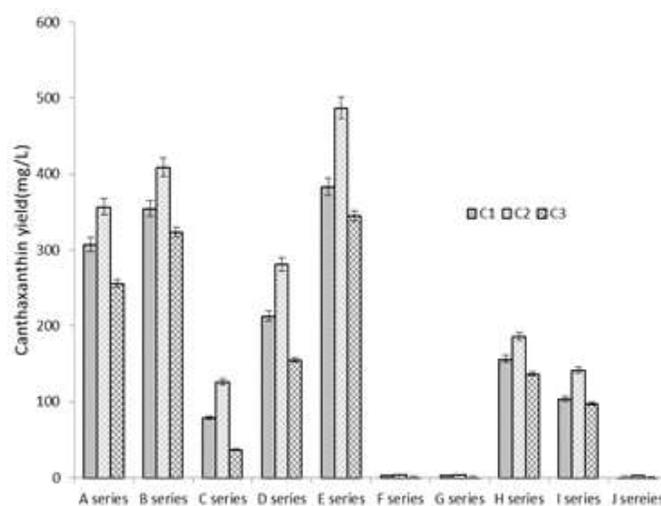


Fig. 1: Yield of canthaxanthin in different customized medium series supplemented with coconut water. C1=tender coconut water, C2=moderate aged coconut water and C3=mature coconut water. Each value represents the mean \pm S.D of three replicates.

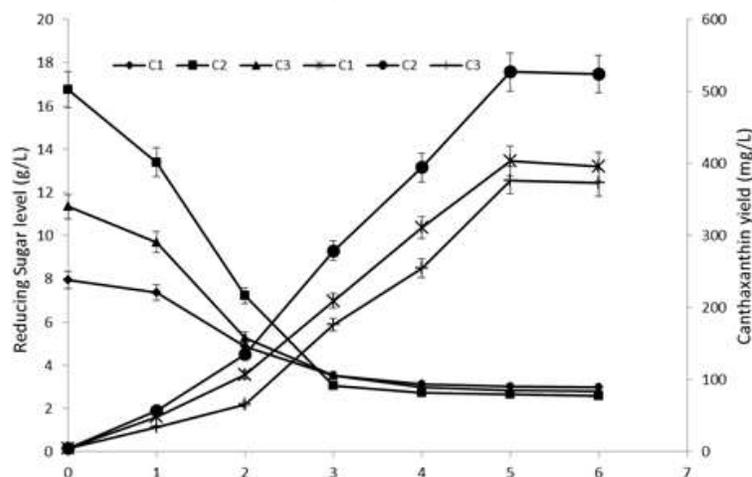


Fig. 2: Reducing sugar utilization during canthaxanthin production. C1=tender coconut water, C2=moderate aged coconut water and C3=mature coconut water. Each value represents the mean \pm S.D of three replicates.

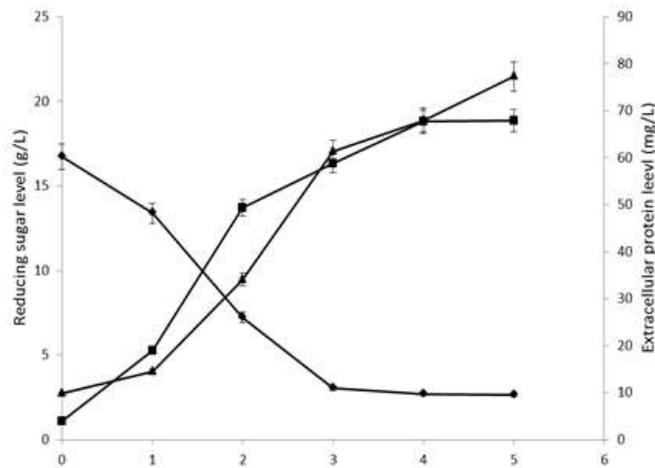


Fig 3: Reducing sugar content along with biomass and extracellular protein content when moderate aged coconut water was supplemented. a) solid black diamond represents reducing sugar level (g/L), b) solid black squares represents microbial biomass yield (g/L), c) solid black triangles represents extracellular protein level during fermentation(mg/L). Each value represents the mean \pm S.D of three replicates.

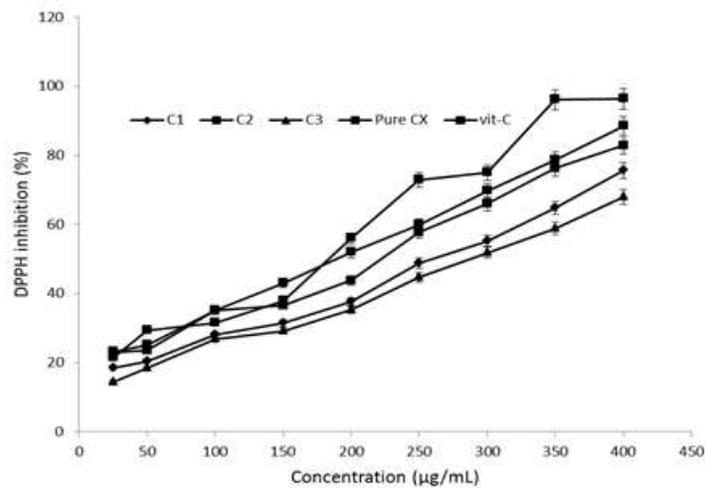


Fig 4: DPPH inhibition by the pigment produced from different aged coconut water supplemented medium. C1=tender coconut water, C2=moderate aged coconut water and C3=mature coconut water, pure CX= commercial canthaxanthin (standard), vit-C= Vitamine C (as positive control). Each value represents the mean \pm S.D of three replicates.

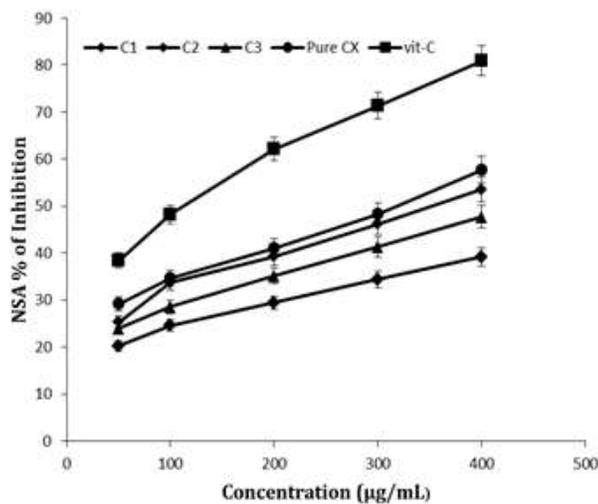


Fig 5: Nitrite scavenging activity of pigment produced from different aged coconut water supplemented medium. C1=tender coconut water, C2=moderate aged coconut water and C3=mature coconut water, pure CX= commercial canthaxanthin (standard). Each value represents the mean ± S.D of three replicates.

TABLE I: Different Medium Formulation with the Established Customised Medium Components Supplemented With Different Types of Coconut Water

Coconut Age	3-4 months (C1)										6-8 months (C2)										10-12 months (C3)									
	A	B	C	D	E	F	G	H	I	J	A	B	C	D	E	F	G	H	I	J	A	B	C	D	E	F	G	H	I	J
Medium symbolized																														
D-(+)-glucose (1.5%)	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	-	-	-
NaCl (0.5%)	+	-	+	+	+	-	+	-	-	-	+	-	+	+	+	-	+	-	-	-	+	-	+	+	+	-	+	-	-	-
Bacteriological peptone (1%)	+	+	-	+	+	-	-	+	-	-	+	+	-	+	+	-	-	+	-	-	+	+	-	+	+	-	-	+	-	-
Yeast extract (0.5%)	+	+	+	-	+	-	-	-	+	-	+	+	+	-	+	-	-	-	+	-	+	+	+	-	+	-	-	-	+	-
Coconut Water	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+