IJPSR (2017), Vol. 8, Issue 6 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 23 November, 2016; received in revised form, 13 January, 2017; accepted, 14 January, 2017; published 01 June, 2017

IMPACT OF DIFFERENT CARBON SUPPLEMENT ON EXTRACTION OF C-PHYCOCYANIN (C-PC) FOLLOWED BY POLY-β-HYDROXYBUTYRATE (PHB) FROM *NOSTOC MUSCORUM*

Vrijesh Kumar Yadav [*1, 2, 3] and Vikash Kumar Yadav [2]

Indian Institute of Technology [1], Kharagpur -721302, West Bengal, India.
Academy of Scientific and Innovative Research (AcSIR) [2], CSIR-National Botanical Research Institute, Rana Pratap Marg, Lucknow - 226001, Uttar Pradesh, India.
State Forensic Science Laboratory (SFSL) [3], Ranchi - 835217, Jharkhand, India.

Keywords:

C-phycocyanin, *Nostoc muscorum*, Carbon source, Mixotrophy, Poly-β-hydroxybutyrate

Correspondence to Author:
Mr. Vrijesh Kumar Yadav

(M. Tech. IIT Kharagpur)
Scientific Assistant (SA)
State Forensic Science Laboratory (SFSL), Ranchi - 835217, Jharkhand, India.

E-mail: yadav.vrijesh@gmail.com

ABSTRACT: The extraction of poly-β-hydroxybutyrate (PHB) and C-phycocyanin (C-PC) from *Nostoc muscorum* revealed that PHB yield after C-PC extraction was not found differ significantly as without C-PC extraction. Under photoautotrophic growth condition, the test cyanobacterium accumulates PHB and C-PC with a maximum yield of 40 and 57 mg l-1 respectively at the stationary phase, *i.e.* on day 21 of incubation. The most significant enhancement in C-PC yield up to 146 mg l-1 was recorded in 0.4 % fructose supplementation. High C-PC and PHB yield was observed in 0.4% fructose supplementation followed by 0.4% glucose supplementation. Here, in this study we show, that PHB yield after C-PC extraction was not affected significantly and C-PC yield was found enhanced under 0.4 % carbon source (fructose and glucose) supplementation. Significant Biomass and PHB yield depicted under supplementation of glucose, fructose, maltose and sucrose was due to boost in growth. Thus, present study demonstrates the extraction of both the products, *i.e.* PHB and C-PC is possible.

INTRODUCTION: Cyanobacteria are being used for the extraction of C-PC and recently also considered as a source of PHB. They are the simplest known photoautotrophic organisms that accumulate PHB as energy storage. Due to photosynthetic consumption of CO_2 by cyanobacteria, they altering earth's atmosphere and leading to the production of oxygen gas [1]. Cyanobacteria produce several bioactive compounds that have antibacterial, antifungal, antiviral, and antialgal properties of pharmaceutical and agricultural significance [2].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.8(6).2469-76

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.8 (6).2469-76

For the production of valuable metabolites several microorganisms have been utilized in the past although the potential of cyanobacterial species for many of the metabolites are largely unexplored [3]. Recently it has been demonstrated that (S) and (R)-3HB (precursor of biodegradable plastic) can be photosynthetically produced from source of sunlight and CO_2 [4]. However, it is well known that PHA is a natural energy and carbon storage product of prokaryote [1].

It have been reported that most of the known cyanobacteria producing polyhydroxy alkanoate (PHAs) approximately up to 6% [5, 6]. Recently it has been reported that under bright sunlight and high cell-density conditions photosynthetic productivity and biomass accumulation enhances in a cyanobacteria [7]. It also has been reported that flux of carbon is also the possible driving force for the biosynthesis of PHA [8].

Cyanobacteria are also being used for extractions of C-phycocyanin [9, 10]. As cyanobacteria have short generation time and photoautotrophic in nature attract the attention to produce PHA and C-PC. Extraction and purification of C-PC has been reported from different cyanobacterial strains [11, 10]. Recently work revealed that C-PC have protective effect against acute lung injury induced by lipopolysaccharide [12]. CPC have therapeutic effect against pulmonary fibrosis [13], also used as food [14], coloran [15], fluorescent dye [16], and antimalarial activity [17]. C-PC also functions in antioxidation [16] and have antitumor [18], immunity enhancement [19], antioxidant and antiproliferative activities [20].

The work on cyanobacteria for PHB extraction has been well reported by many researchers [21, 22, 23]. More recently, PHB accumulation found up to 46% under chemo-heterotrophy and phosphate limitation [23]. Recently there is a report for improvement of solar energy conversion efficiency by enhancing natural photosynthesis [24], if it happens it would be very useful to increase C-PC and PHB production. Specific objectives of the present study, is to explore the extraction of C-PC followed by PHB extraction from the *Nostoc muscorum* and also the impact of carbon sources on C-PC and PHB production.

MATERIALS AND METHODS:

Organism and growth conditions: Established culture of *Nostoc muscorum* Agradh was maintained in complete BG-11 medium[25]. The *Nostoc muscorum* culture was maintained in a controlled culture conditions 28 ± 2 °C, pH 8.5, under a photoperiod of 14:10 h at light intensity of 75 µ mol photon m-2 s -1 PAR.

Estimation of dry cell weight (dcw): Estimation of dry cell weight was done accordingly [26]. Acetone extraction method was followed for the extraction of C-PC. Cell culture of *N. muscorum* was taken in a centrifuge tube and centrifuged (5000 rpm, 10 min), discarded the supernatant followed by addition of 20 ml (80% acetone). Such an obtained pellet Kept for incubation (over night, 4 °C), centrifuged (5000 rpm, 10 min), discarded the supernatant added 20 ml of distilled water and kept at 50 °C for 30 min. To get the supernatant containing the crude phycobiliproteins centrifuged the tube (5000 rpm, 10 min). After phycocyanin extraction processed the same pellet for PHB extraction in methanol at 4 ^{0}C (overnight) for removal of other pigments. Centrifuged (5000 rpm, 10 min) discarded the supernatant and dried the pellet (60 ^{0}C). The hot chloroform extractions were performed for PHB and then precipitate in diethyl ether, centrifuged (5000 rpm, 20 min) to get the pellet and the same was dissolved in hot chloroform.

Estimation of C-PC: The C-PC estimation was performed using the UV-Vis absorbance spectra (250-820 nm) obtained from a spectrophotometer (Lambda 25 UV/Vis). The C-PC yield and purity ratio (R) estimated accordingly [27, 28]. C-PC concentration (mg ml-1) and purity ratio (R) was calculated by using the following formula: C-PC (mg ml-1) = (A620 – 0.474 × A650)/5.34 Purity ratio (R) = A620 / A280

Detection and confirmation of poly-β-hydroxybutyrate (PHB): The spectrophotometric assay was performed following Law and Slepecky (1961) [29]. The sample containing the polymer in chloroform was transferred to a clean test tube. The chloroform was evaporated and concentrated H_2SO_4 (10 ml) was added, heated in a boiling water bath (10 min). Detection and confirmation of PHB was also done gas-chromatographically following Riis and Mai (1988) [30] using a Gas Chromatograph (Perkin Elmer, Shelton, CT, USA). The extracted polymer isolated from 20 ml biomass suspended with 2ml 1,2-dichloroethane, 2ml propanol containing hydrochloric acid (1:4, v/v), standard solution (4%, w/v) the samples were shaken for 30 sec. After 24 h, organic phase was directly analysed. Mixotrophic growth conditions were achieved by supplementing the nutrient media with 0.2 and 0.4% of glucose, fructose, maltose, sucrose, and acetate at the time of incubation.

RESULTS: Detection and confirmation of C-PC and PHB from *N. muscorum* grown under photoautotrophic mode were analyzed. (**Fig. 1A**).

The polymer extracted from *N. muscorum* and the standard PHB after acid digestion represented in **Fig. 1B**, both the spectra depicted complete matching. PHB and C-PC content with reference to growth under batch mode is presented in **Fig. 1C**.

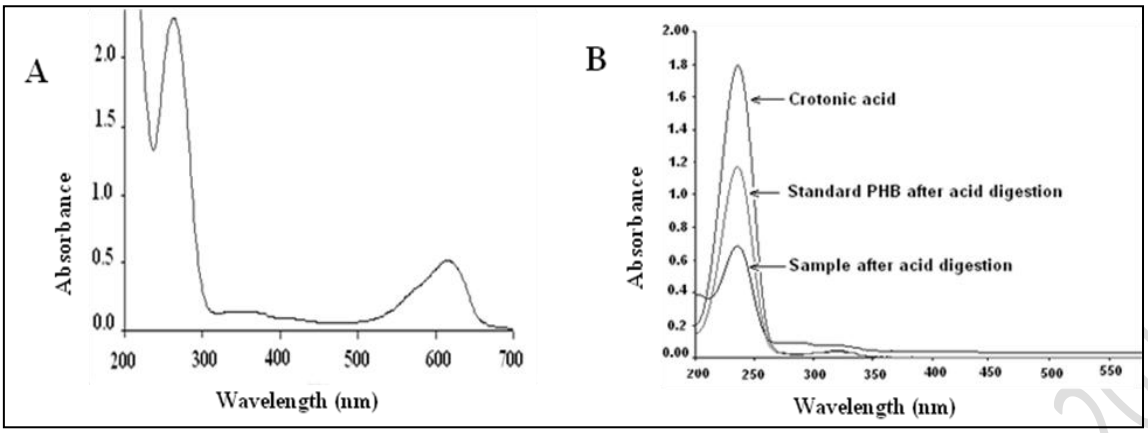

FIG. 1: (A) ABSORPTION SPECTRUM OF *C-PC* (B) SPECTRA OF PHB EXTRACTED FROM *N. MUSCORUM*

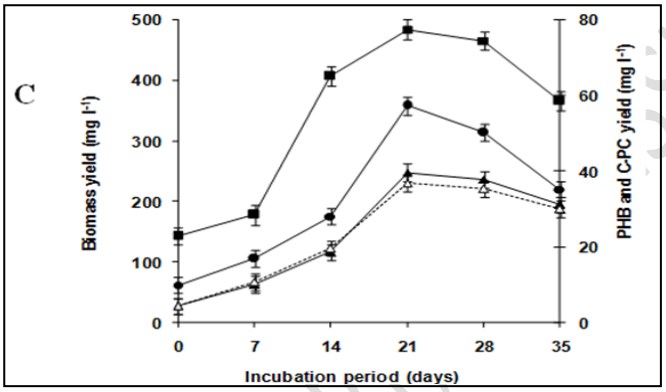

FIG. 1: (C) PHB AND *C-PC* ACCUMULATION IN *N. MUSCORUM* WITH REFERENCE TO GROWTH IN PHOTOAUTOTROPHIC CONDITIONS (■) Biomass, (●) *C-PC*, (▲) PHB and (Δ) PHB followed by *C-PC* extraction

Growth curve of *N. muscorum* revealed a lag of 7 days followed by the logarithmic phase and the stationary phase on day 21. The maximum PHB and C-PC accumulation was observed at the stationary phase, i.e. on day 21 (40 mg l^{-1}) and (57 mg l^{-1}, **Fig. 1C**) respectively. PHB yield after C-PC extraction was not found differ significantly without C-PC extraction (**Fig. 1C**). Impact of carbon source supplementation (acetate, fructose, glucose, maltose and sucrose) on PHB and C-PC studied. Supplementation of acetate, fructose, glucose, maltose and sucrose stimulate PHB yield (**Fig. 2-6**). Maximum C-PC and PHB yield was observed on day 21 of incubation.

Effect of acetate: Cultures supplemented with acetate depicted significant rise in biomass and PHB content. Biomass yield reached only upto 512.2 mg l^{-1} under 0.4% acetate supplementation as compare to 484 mg l^{-1} on day 21 of incubation (**Fig. 2A**)

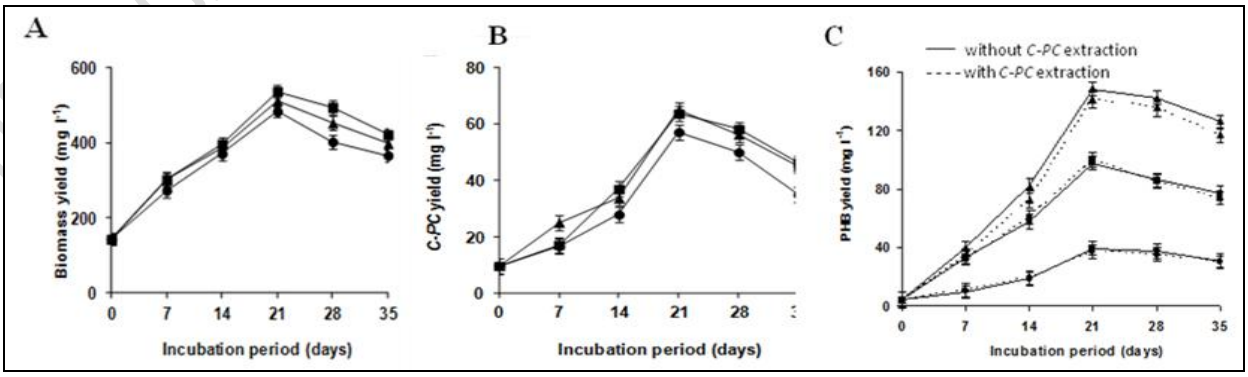

FIG. 2: EFFECT OF ACETATE SUPPLEMENTATION ON BIOMASS, C-PC & PHB YIELD OF *N. MUSCORUM*. (●) Control, (■) 0.2% acetate and (▲) 0.4% acetate

An increase up to 65 mg l^{-1} (C-PC) and 141 mg l^{-1} (PHB) yield revealed with 0.4% acetate supplementation, on day 21 of incubation (**Fig. 2B** & **C**). The PHB yield (141 mg l^{-1}) obtained in C-PC extracted sample was found comparable with the PHB yield obtained from the sample without C-PC extraction (148.9 mg l^{-1}) **(Fig. 2C**).

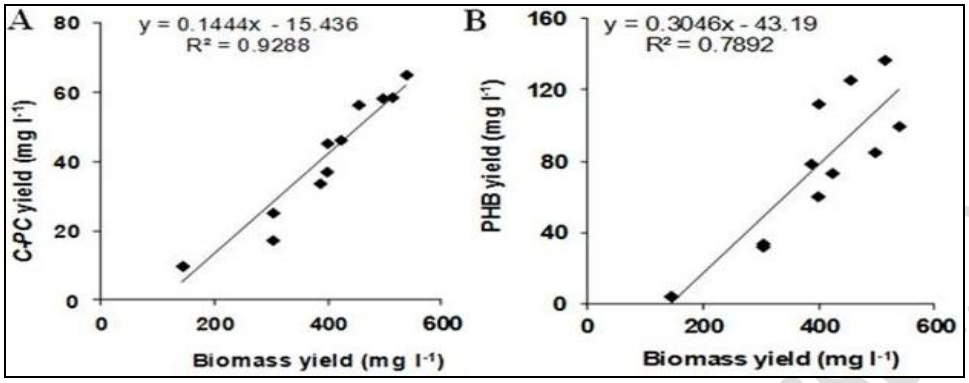

FIG 2.1: RELATIONSHIP BETWEEN (A) BIOMASS AND C-PC YIELD (B) BIOMASS AND PHB YIELD OF *N. MUSCORUM* UNDER ACETATE SUPPLEMENTATION

Relationship of C-PC and biomass yield and PHB and biomass yield shown in **Fig 2.1A** & **B,** where the C-PC yield with biomass are more correlated than PHB yield with biomass.

Effect of fructose: similarly, fructose supplementation enhances biomass C-PC and PHB yield (**Fig. 3A**).

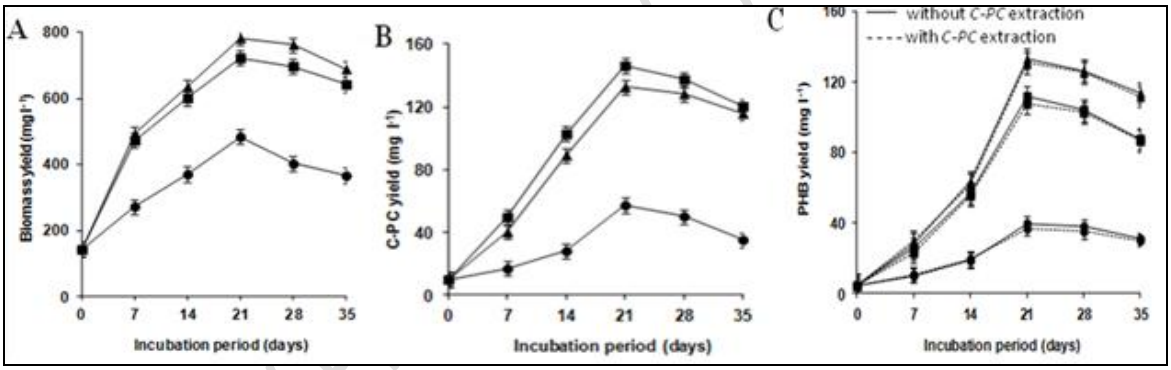

FIG. 3: EFFECT OF FRUCTOSE SUPPLEMENTATION ON BIOMASS, C-PC & PHB YIELD OF *N. MUSCORUM*. (●) Control, (■) 0.2% fructose and (▲) 0.4% fructose

Biomass yield reached up to 783 mg l^{-1} was in 0.4% fructose supplemented culture. C-PC yield reached up to 146 mg l^{-1} in 0.4 % fructose supplementation followed by 132 mg l^{-1} in 0.2% fructose supplementation day 21 of incubation (**Fig. 3B**). After C-PC extraction, PHB yield of 132 mg l^{-1} in 0.4 % fructose and 107 mg l^{-1} in 0.2% fructose, respectively were recorded (**Fig. 3C**).

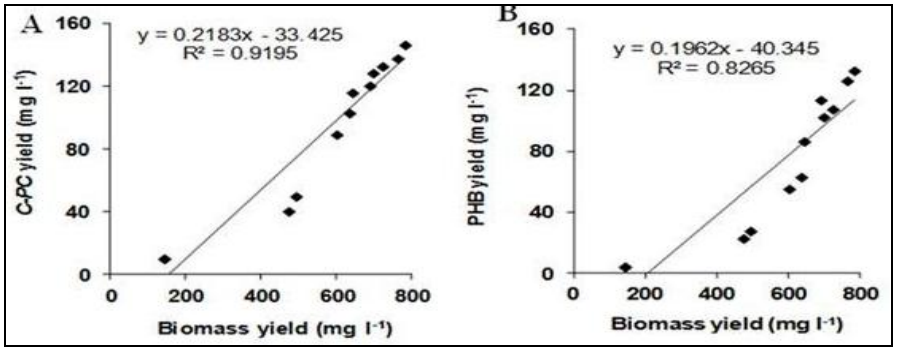

FIG 3.1: RELATIONSHIP BETWEEN (A) BIOMASS AND *C-PC* YIELD (B) BIOMASS AND PHB YIELD OF *N. MUSCORUM* UNDER FRUCTOSE SUPPLEMENTATION

When this yield was compared with PHB yield without C-PC extraction (130 mg l^{-1} in 0.4 % fructose and 111 mg l^{-1} in 0.2% fructose supplementation, respectively), biomass and C-PC yield was more significant than biomass vs PHB yield (**Fig. 3.1** A and **B**).

Effect of glucose: Under glucose supplementation, total biomass content increased up to 652 mg l^{-1} in 0.4% glucose supplemented culture, which was 35% higher against 484 mg l^{-1} (**Fig. 4**). The enhancement of PHB pool was observed (132 mg l^{-1} and 108 mg l^{-1}) in 0.4% and 0.2% glucose-supplemented cultures, respectively on day 21 of incubation (**Fig. 4C**).

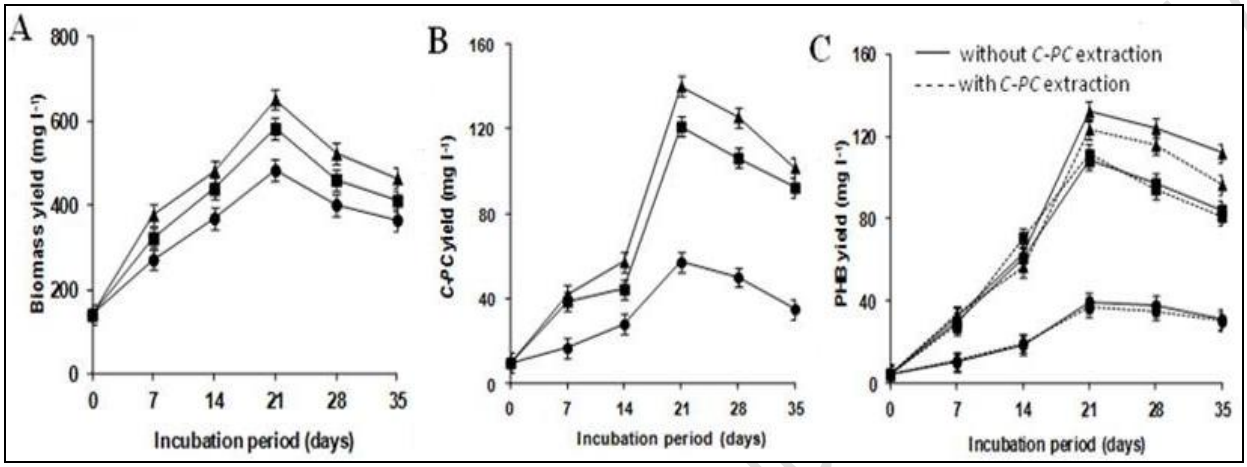

FIG. 4: EFFECT OF GLUCOSE SUPPLEMENTATION ON BIOMASS, *C-PC* & PHB YIELD OF *N. MUSCORUM*. (●) Control, (■) 0.2% glucose and (▲) 0.4% glucose

When PHB and C-PC was extracted simultaneously, PHB yield was found 123 mg l^{-1} and 111 mg l^{-1} under 0.4% and 0.2% glucose-supplementation, respectively. C-PC yield increased up to 140 mg l^{-1} under 0.4% glucose supplementation which was 2.5 fold higher than the control condition (57 mg l^{-1}). C-PC accumulation was significantly correlated to biomass yield (**Fig. 4.1 A**).

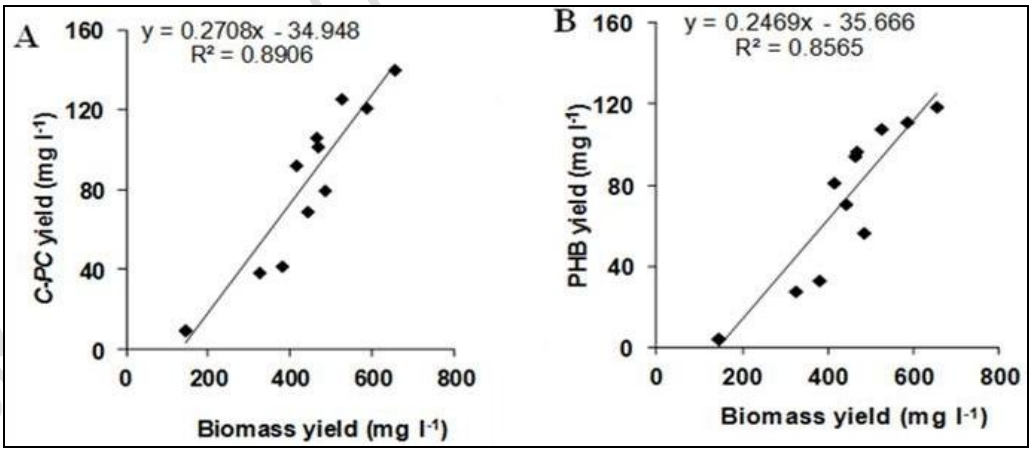

FIG 4.1: RELATIONSHIP BETWEEN (A) BIOMASS AND *C-PC* YIELD (B) BIOMASS AND PHB YIELD OF *N. MUSCORUM* UNDER GLUCOSE SUPPLEMENTATION

Effect of maltose and sucrose: Significant enhancement in biomass yield up to 35% and 32% were observed under 0.4% maltose and sucrose supplementation respectively, on day 21 of incubation when compared against the control condition (**Fig. 5A** and 6A). Significant enhancement of PHB yield was obtained under maltose and sucrose supplementation, which was however, much lower than glucose, fructose and acetate supplementation (**Fig. 2A**, **3A & 4A**).

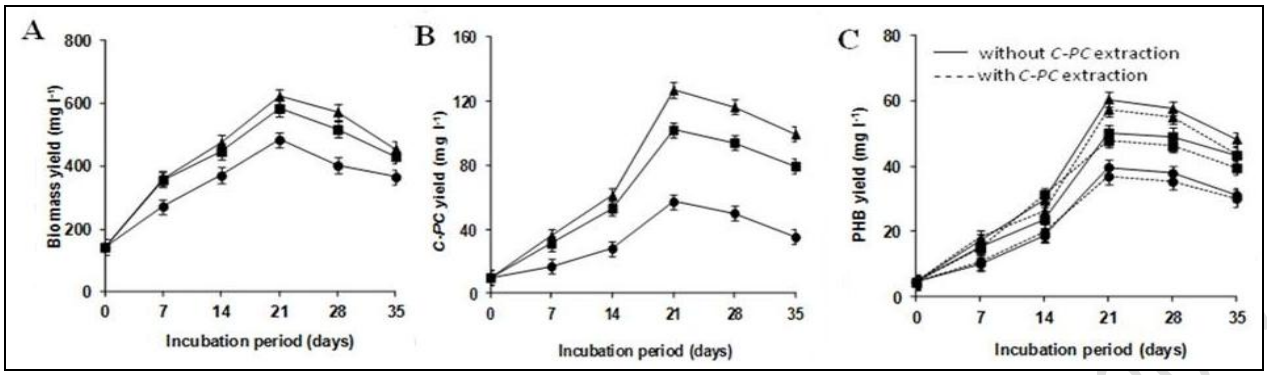

FIG. 5: EFFECT OF MALTOSE SUPPLEMENTATION ON BIOMASS, *C-PC* & PHB YIELD OF *N. MUSCORUM*. (●) Control, (■) 0.2% maltose and (▲) 0.4% maltose

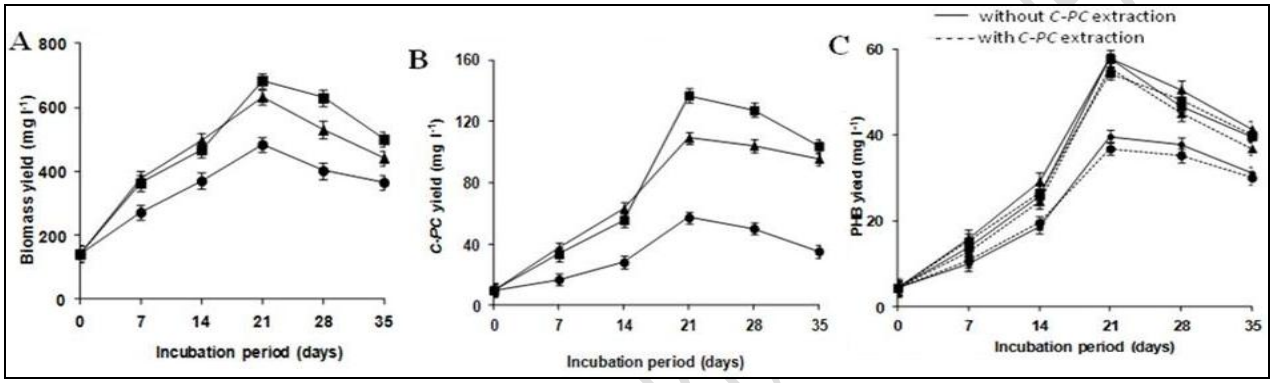

FIG. 6: EFFECT OF SUCROSE SUPPLEMENTATION ON BIOMASS, *C-PC* & PHB YIELD OF *N. MUSCORUM*. (●) Control, (■) 0.2% sucrose and (▲) 0.4% sucrose

Maltose supplementation enhanced the C-PC yield of 127 mg l^{-1} under 0.4% maltose supplementation on day 21 of incubation (**Fig. 5B**). With sucrose supplementation, C-PC content increased, attaining a maximal value 136 mg l^{-1} (0.2% sucrose) during stationary phase (**Fig. 6B**). The yield of C-PC and biomass and PHB and biomass are comparable (**Fig. 6.1A** & **Fig. 6.1B**).

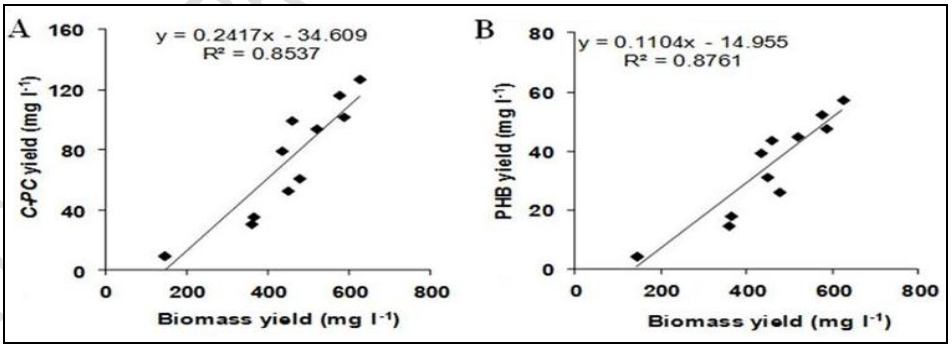

FIG 6.1: RELATIONSHIP BETWEEN (A) BIOMASS AND *C-PC* YIELD (B) BIOMASS AND PHB YIELD OF *N. MUSCORUM* UNDER SUCROSE SUPPLEMENTATION

Thus, Biomass, PHB and C-PC yield, from *N. muscorum* under various conditions revealed that glucose and fructose are more suitable substrates.

DISCUSSION: The Cyanobacteria (photoautotrophic) is promising eco-friendly microorganism as the 'green house gas' is photosynthetically converted into biodegradable plastics and C-PC by utilizing sunlight as the energy source. The positive impact of acetate, fructose, glucose, maltose and sucrose was found on PHB accumulation. Interestingly, in the acetate-supplemented cultures, an insignificant correlation with growth was established ($r = 0.883$, $P > 0.05$).

The availability of PHB precursor i.e. acetyl-CoA causes the enhanced PHB pool up to 141 mgL^{-1} under 0.4% acetate-supplemented cultures [31, 32]. The availability of carbon influenced the accumulation of C-PC in *N. muscorum*. Carbon supplementation resulted in accumulation of relatively high cellular C-PC contents and maximum was found 146 mg l^{-1} under 0.4% fructose supplemented condition.

The growth and biomass concentration increases after the addition of glucose and acetate in Spirulina platensis culture [33]. More recently, much attention has been drawn to the potential use of cyanobacteria to extract high value chemicals. In this study, maximum C-PC and PHB yield were observed in 0.4% fructose supplementation followed by 0.4% glucose supplementation. Fructose has maximum stimulatory effect on simultaneous C-PC and PHB yield.

CONCLUSION: PHB yield without C-PC extraction was not found to differ significantly from PHB yield with C-PC extraction simultaneously. The C-PC content increased up to 146 mgL^{-1} under 0.4% fructose supplementation followed by 140 mgL^{-1} under 0.4% glucose supplementation. Thus demonstrating the extraction of both PHB and C-PC is possible.

ACKNOWLEDGEMENTS: VKY1 is grateful to Indian Institute of Technology Kharagpur for providing fellowship support and also thankful to N. Mallick (Agricultural and Engineering Department Indian Institute of Technology Kharagpur) to provide facility to conduct the work.

CONFLICT OF INTEREST: The authors do not have any conflict of interest.

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How to cite this article:
Yadav VK and Yadav VK: Impact of different carbon supplement on extraction of c-phycocyanin (C-PC) followed by poly-β-hydroxybutyrate (PHB) from *Nostoc muscorum*. Int J Pharm Sci Res 2017; 8(6): 2469-76.doi: 10.13040/IJPSR.0975-8232.8(6).2469-76.