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Ultrasound assisted three phase partitioning (UA-TPP): A novel technique developed for extraction of astaxanthin from *Paracoccus* NBRC 101723

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ABSTRACT Astaxanthin (AX) is an orange red colored carotenoid found largely in the marine habitat. Owing to its powerful antioxidant properties it has a wide range of applications in nutraceutical, cosmetics, food, and feed industries, making it a commercially attractive biomolecule. Its intracellular accumulation in bacterial cells of Paracoccus NBRC 101723 in minute quantities

necessitates an efficient recovery process. The present study investigates development of a combined process using ultrasound extraction for lysing the bacterial cells followed by three phase partitioning (TPP) for the extraction of AX. The operational parameters for ultrasonication were optimized with amplitude of 100% for 20 s (wet biomass) and 60 s (dried biomass). The ultrasonic extraction was significantly affected by solid to solvent ratio (1:2), and distance between the base of the extraction vessel and the tip of the probe (15 mm). Pretreatment of the dried biomass (particle size 0.8 µm) with 70% acetone at 70 °C for 25 min resulted in maximum ultrasonic AX extraction at 1035 µg/g of dried biomass. The wet biomass was preferred over dried as it was easier and rapid to extract AX from it. The conditions for TPP were optimized at ammonium sulphate loading (40%), biomass to t-butanol ratio (1:0.75), temperature (40 °C), and extraction time (30 min).This approach of ultrasound assisted TPP resulted in maximum recovery of AX (428 µg/g of wet biomass) in comparison to conventional solvent extraction (317 µg/g).

Keywords: Astaxanthin, *Paracoccus* NBRC 101723, ultrasonication, Three phase partitioning

INTRODUCTION Astaxanthin (3, 3´-dihydroxy-ß, ß-carotene 4, 4´-dione), a ketocarotenoid most commonly found in marine animals, is responsible for the red coloration of crustaceans, shellfish and flesh of salmonoids (Fraser et al. 1998). Its role as a free radical scavenger, skin protectant, anti-carcinogenic and as an enhancer of immune system justifies its applications in foods, pharmaceuticals and cosmetics (Lorenz et al. 2000). Its chemical synthesis involves a multistage process and its acceptability amongst the consumers is questionable (Ernst 2002). Therefore its production from natural sources such as microorganisms is in demand. AX production from *Phaffia rhodozyma* and *Hematococcus pluvialis* is well known, but commercial application is hampered due to their low growth rate and thick cell walls (Kim et al. 2006). Bacterial source of AX can be commercially profitable owing to its smaller batch time and ease of extraction. Various researchers have reported different *Paracoccus* species for AX production (Lee et al. 2004; Yokoyama et al. 1994). *Paracoccus* MBIC 11043 formerly known as *Agrobacterium auarantiacum* produces 3S, 3S´ form of AX and seems to be promising for industrial production (Yokoyama et al. 1994). Recently, we reported enhanced AX production from *Paracoccus sp*. NBRC 101723 (MBIC 01143) on supplementation of fermentation medium with TCA intermediates and cofactors (7).

AX is an intracellular carotenoid which is sensitive to oxygen, heat, and light. This poses challenges in its extraction from microbial biomass. Several reports have been published earlier on AX extraction from algae and yeast. These include autoclaving under acidic conditions (Xiao et al. 2009) and microwave assisted extraction (Zhao et al. 2009) which could overcome the difficulties that arise due to thick encysted cell wall of *Heamatococcus* and rigid cell wall of *Phaffia* (Xiao et al. 2009). In case of bacteria, such harsh treatments are not necessary due to simpler structure of its cell wall making it amenable to disruption by simpler disruption techniques. In the present study, we report the use of high-intensity, low-frequency ultrasound waves to bring about the disruption of bacterial cells followed by extraction of AX using three phase partitioning

METHODOLOGIES, RESULTS AND DISCUSSION The bacterial strain used for production of astaxanthin was *Paracoccus sp*. NBRC 101723 (formerly available as MBIC 01143, also classified as *Agrobacterium aurantiacum*) was procured from Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE), Chiba, Japan. The production medium used was- glycerol (2 g/L), soy peptone (39.3 g/L), NaCl (22.3 g/L) at pH 7.5 ± 0.2. The fermentation was carried out at 20°C for 4 days (7).

Ultrasonic extraction of AX from wet biomass For the ultrasonic extraction of AX from wet biomass ultrasonic device of 400 W and 24 kHz (UP400S, Hielscher, Germany) was used equipped with a H3 sonotrode (made of titanium, tip diameter 3 mm, length 100 mm). The bacterial cells cultivated were harvested by centrifugation of fermentation broth at 10000 *g* for 20 min. The cell pellet (1 g) was then suspended in acetone (10 mL) and subjected to ultrasonication using ultrasonic power of amplitude from 10 to 100 %. The extracted AX was analyzed by HPLC (Agilent, USA) equipped with a Hypersil C-18 column (4.6 mm × 150 mm × 3 µm, Phenomenex, Torrance, Canada). A mobile phase of methanol: water (95:5) with a flow rate of 0.8 mL/min at 25 ± 2°C was used for elution. Detection was carried out with a visible detector at 476 nm. The amount of AX was estimated from the calibration curve obtained with an authentic sample (7). A standard curve of 98% pure AX was plotted using concentration range 10 - 100 µg/mL (R^2 = 0.998). All the experiments were carried out in triplicates. The results were expressed as mean ± standard deviation of three determinations. The results were analyzed by Student T test. P value < 0.05 indicated statistically

significant differences among samples. Ultrasound extraction at 100% amplitude resulted in maximum AX extraction of 347 ± 3 µg/g in shortest extraction time of 20 s (Fig. 1).

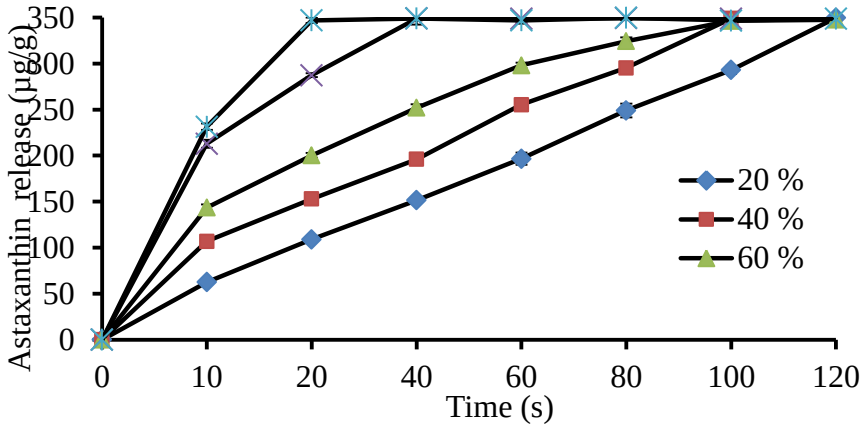


FIG. 1. Ultrasonic release of AX from wet biomass of *Paracoccus sp*. NBRC 101723: effect of amplitude and time interval

We further investigated the effect of other namely wet biomass: solvent ratio, distance between base of extraction vessel and tip of probe, volume and shape of extraction vessel (Table. 1).

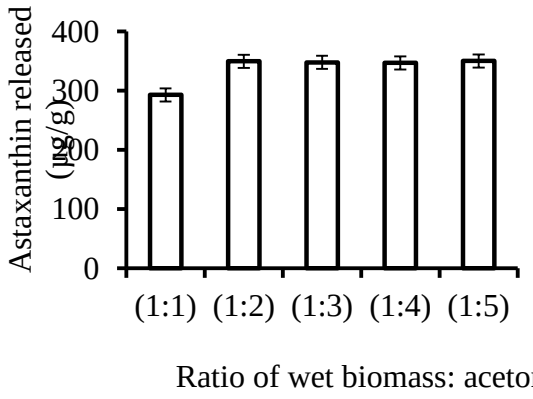
TABLE 1 Effect of wet biomass: solvent ratio, distance between base of extraction vessel and tip of probe, volume and shape of extraction vessel

Wet biomass: solvent (Ratio)	Distance between base of extraction vessel and tip of probe (mm)
(1:1)	3
(1:2)	9
(1:3)	15
(1:4)	21
(1:5)	
Volume (mL)	**Shape of vessel**
250	Round
100	Flat
50	Conical

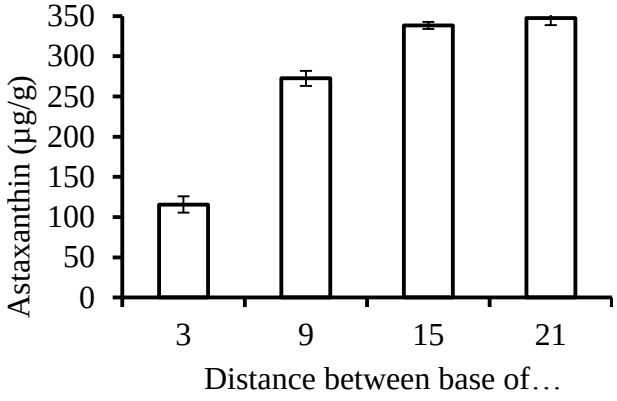
Ratio of solid to solvent was varied from 1:1 to 1:5 (Fig. 2a).There was a significant increase in AX release above a ratio of 1:2. At a ratio 1:1 or lower, AX extraction was lowered probably due to poor propagation of ultrasound waves though the thick slurry of biomass (20). Kanthale et al. (21) mapped the working of ultrasonic horn. Their results indicated the intensity of the ultrasonic power to decrease as the location moves away from the source of the ultrasonic waves reducing the penetration power of waves.

Parameters such as distance between base of extraction vessel and tip of probe, size and shape of vessel will therefore influence the performance of ultrasonication. Effect of each of this parameter on AX extraction was studied individually **(**Fig. 2b, c, d). Results obtained in the present study support the findings of Kanthale et al. (21). The extraction of AX lowered with an increase in the distance between the base of vessel and the tip as the ultrasonic intensity which rapidly decreases axially from the ultrasonic probe (22). A distance of 15 mm from the base of extraction vessel to the tip of probe was optimum for AX extraction. The extraction vessel with lowest size (50 mL) enhanced the interaction between the particles and the cavitation bubbles bringing out the maximum extraction (22). The different shapes of vessels did not show any significant difference in extraction.

a)



b)



c)

d)

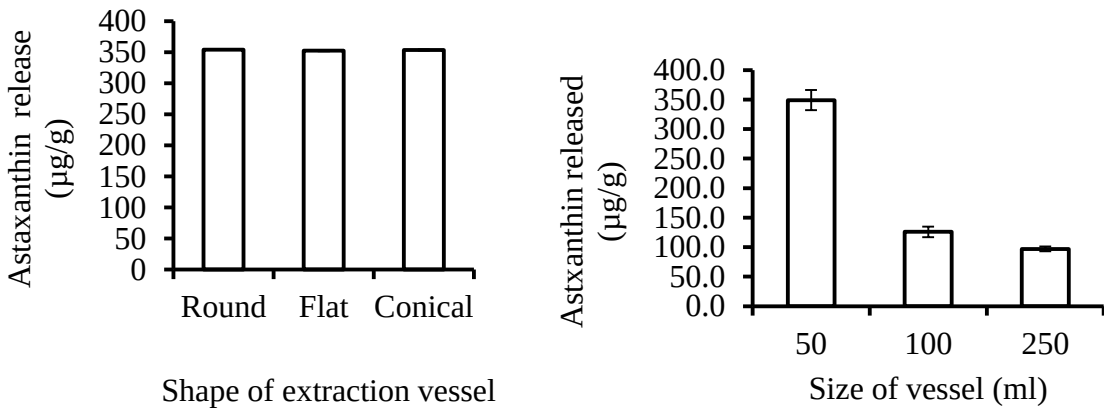


FIG. 2. Effect of wet biomass: solvent ratio (a), distance between base of extraction vessel and tip of probe (b), shape (c), and size (d) of extraction vessel

Ultrasonic extraction from dried biomass Ultrasonic extraction of AX from dried biomass was also studied and the various parameters were optimized. It was observed in the initial experiments that due to compact nature of the dried biomass it had to be pretreated with aqueous solvent at a higher temperature in order to make it more susceptible to ultrasonication. The vacuum oven dried biomass (1 g) was treated with 60-100% acetone at 50°C for 10 min and then subjected to ultrasound extraction. 70% acetone extracted the maximum AX of 649 ± 8 µg/g as compared to 61.9 ± 4 µg/g with 100% acetone. The time and temperature for pretreatment was optimized to 70°C for 25 min by treating the dried biomass for varying temperature (30-90°C) from 5 to 25 min using 70% acetone at 1:2 ratio. To study the effect of particle size, the dried biomass was ground into particles and the mixture was sieved through different sieves (A.S.T.M.E-11 Specification, VWR international, USA) to get particles of size 9.6, 4.8, 2.4, and 0.8 mm. The particle size of 0.84 mm gave the best extraction (Fig. 2c). Smaller size increases the surface area which enhances the contact between acetone and biomass eventually leading to enhanced and faster mass transfer. The effect of drying conditions was studied by drying the wet biomass using freeze drying, vacuum oven drying and hot air oven drying (35, 60, and 80 °C). The biomass was dried till a constant weight was attained. The AX extracted was analyzed by HPLC. Comparable results were obtained when the biomass was dried by freeze drying (-47 °C), vacuum oven drying (30°C) and hot air oven drying (35°C), while AX reduced to almost 70% when dried at 60 and 80°C (Fig. 5). Since use of dried biomass for

ultrasonication required additional steps of drying and pretreatment, wet biomass was the preferred choice for TPP.

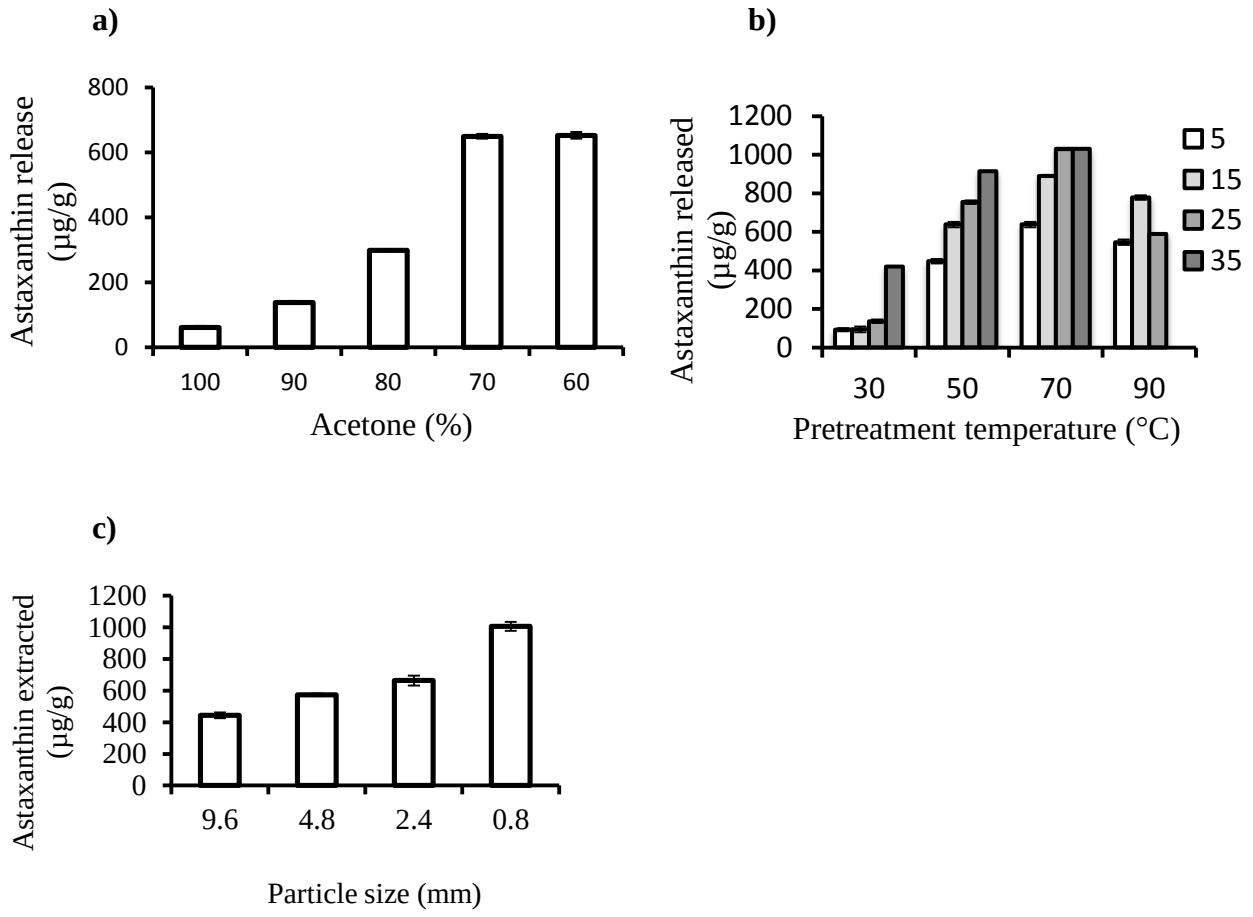


FIG. 3. AX release from dried biomass using varying concentration of acetone at 50 °C for 10 min (a), on pre-treatment with 70% acetone at different temperatures for various time intervals (b), on using different particle size of dried biomass (c)

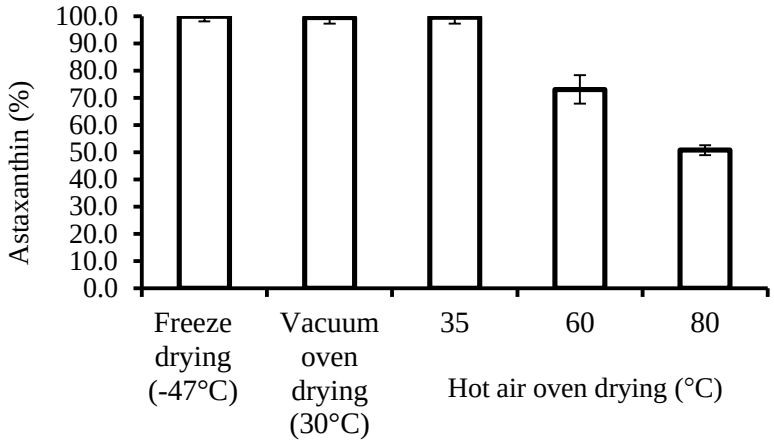


FIG. 4. Effect of various drying methods on AX content form dried biomass

AX recovery using three phase partitioning The ultrasound treated wet biomass was used further for TPP. For TPP, a slurry of ammonium sulphate and wet biomass was prepared and vortexed. *t*-Butanol was added and the mixture was left on rocker for extraction for 1 h. The mixture was centrifuged at 10000 *g* for 15 min for formation of three phases. Upper organic layer containing the extracted AX was collected and analysed by HPLC. The concentration of ammonium sulphate was selected by screening 10 to 60% (w/v) of wet biomass out of which 40 to 60% gave the highest recovery of AX (Fig. 2a). The slurry to *t*-butanol ratio was varied from 1:0.5 to 1:2, a ratio of 1:0.75 extracted maximum AX of 367 ± 12 µg/g. For optimization of incubation temperature, the mixture was incubated at different temperature from 10 to 80°C. 40 and 60^{o}C significantly increased extraction of AX beyond which there was slight reduction (Fig. 2c). Using optimized 40% ammonium sulphate loading and 1:0.75 biomass to *t*-butanol ratio, finally the time of extraction was optimised by varying the time from 15, 30, 45 to 60 min. An incubation time of 30 min showed highest recovery of AX extraction (Fig. 2d). To compare effect of UA-TPP with TPP alone on AX extraction, wet biomass untreated with ultrasound was subjected to TPP at the optimized conditions. The AX extracted was analyzed by HPLC.

a) **b)**

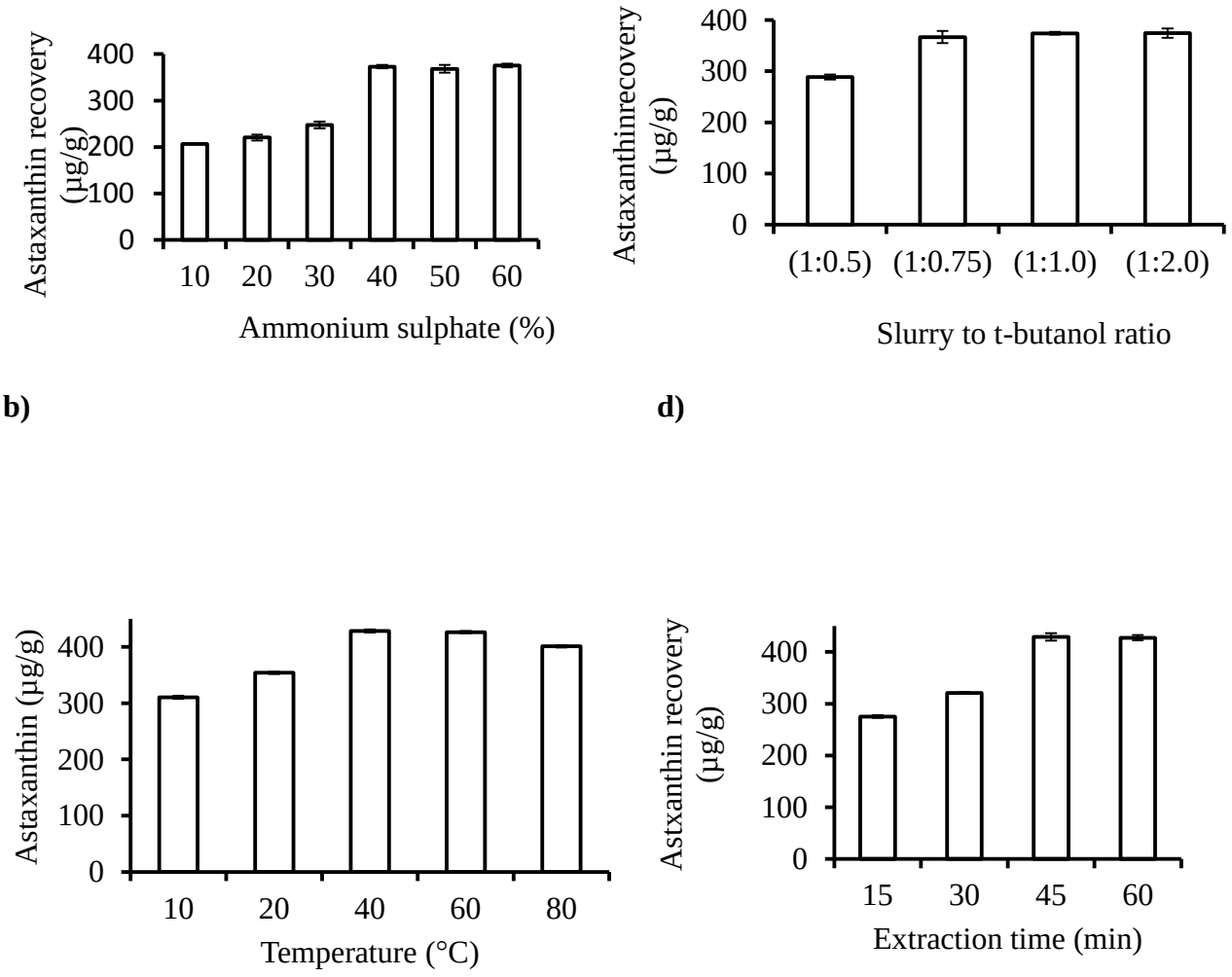


FIG. 5. Effect of ammonium sulphate (a), slurry to *t*-butanol ratio (b), temperature (c) and time (d) on AX recovery by TPP

For the conventional solvent extraction, the harvested cells were suspended in acetone and vortexed. The mixture was centrifuged at 10000 *g* for 5 min. The supernatant containing extracted AX was collected and the pellet was reextracted with acetone. The supernatants collected from three cycles of extraction were pooled for analysis of AX content by HPLC (19). A comparison between the ultrasound, TPP, UA-TPP, and conventional extraction shows AX extracted from UA-TPP to be 428 ± 3 µg/g which is highest than any other technique used alone (Fig 6).

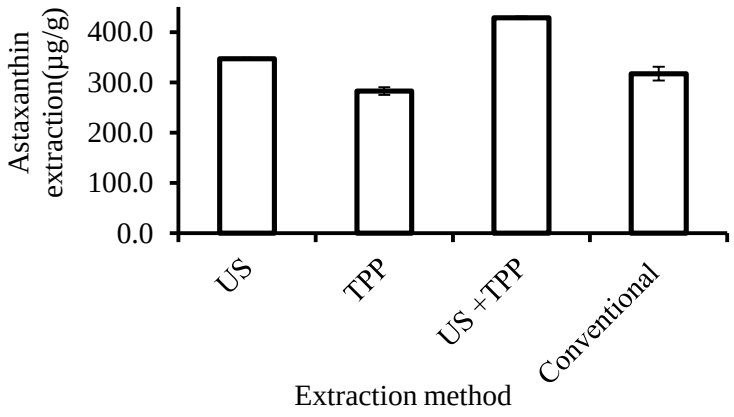


FIG. 6. Comparison of AX recovery using ultrasound extraction, TPP, US TPP, and conventional extraction

CONCLUSION

The combination of ultrasound and TPP was not only simple but also very efficient technique to extract AX from the bacterial biomass of *Paracoccus* NBRC 101723. Evaluation of operating variables for ultrasonication proved sonication time, size, and type of extraction vessel along with nature, particle size, and pretreatment temperature to significantly affect the ultrasonic release of AX from biomass. Ultrasonication with wet biomass was preferred over dried biomass for rapid and efficient extraction of AX. Further three phase partitioning was found to be a useful extraction technique in combination with ultrasonication. This combined strategy gave 37% higher AX recovery than the conventional solvent extraction.

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