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Fermentation of red dragon fruit (*Hylocereus polyrhizus*) for betalains concentration

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Abstract

Red dragon fruits (RDF) contain high levels of health-promoting betalains but its bioavailability in plasma is low (<1.0%). Therefore, fermentation technique was adopted to improve the yield of betalains presenting in fermented red dragon fruit drink (FRDFD) via concentration. Fermentation of RDF was carried out with autochthonous strains. The objectives of present study were to evaluate 1) the effects of fermentation duration 2) white refined cane sugar to flesh ratio and 3) types of sugar on betalainic (betanin, isobetanin) and non-betalainic phenolic compounds in FRDFD using HPLC-DAD, total phenolic content (TPC) and total flavonoid content (TFC) assay. Results indicated that all fermentation parameters showed a significant effect ($p<0.05$) on the yields of betalainic (betanin, isobetanin) and non-betalainic phenolic compounds in FRDFD. The best fermentation parameters were 7 days fermentation at 10% white refined cane sugar to flesh ratio. The highest concentration of betanin, TPC and TFC achieved were 131.68 g/L, 1136.85 mg GAE/100mL and 10.39 mg CE/100mL respectively. The concentration of betanin obtained through best fermentation parameters (131.68 g/L) in present study has increased nine-fold compared to non-optimized fermentation (14.23 g/L). This indicated that fermentation is a potential economic processing technique to concentrate bioactive compounds present in functional drinks.

Introduction

Hylocereus polyrhizus or commonly known as red dragon fruit has been cultivated on a large scale in Malaysia covering 563.2 hectare of plantation area across all states in Malaysia (Department of Agriculture Malaysia, 2015). The increasing production of this fruit is attributed to its high demand and good price in both local and international markets (Zainudin and Hafiz, 2015). This is likely attributed to the antioxidant properties of betalain that found in red dragon fruit that make it highly appealing to the health conscious consumers (Nurliyana *et al.*, 2010).

Betacyanins (betanin, isobetanin, phyllocactin and hylocerenin) which is a sub-group of functional compound betalain has been identified as the main contributing pigment for its deep purple colored pulp appearance in red dragon fruit, with structures as shown in Figure 1 (Rebecca *et al.*, 2010). The potential health benefits of betanin demonstrated in earlier reports are attributed to its free radical scavenging activity of reactive oxygen species (Esatbeyoglu *et al.*, 2014), protection of low density lipoprotein

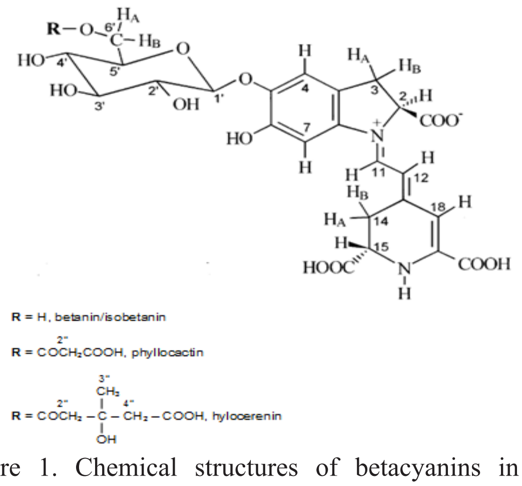

Figure 1. Chemical structures of betacyanins in *Hylocereus polyrhizus*.

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against oxidation (Tsai *et al.*, 2011), prevention of DNA-damage (Esatbeyoglu *et al.*, 2014), induction of antioxidant, gene regulatory activity (Krajka-kuz *et al.*, 2013) and anti-inflammatory activity (Esatbeyoglu *et al.*, 2014). Being one of the plant sources with high amount of betalain which mainly contributed by betanin, red dragon fruit shows great potential as functional food to exhibit desirable bioactive properties (Gengatharan *et al*., 2016a).

Despite having the potential benefits, the low bioavailability of betanin has to be addressed. The bioavailability of betanin is rather low with only 0.5% to 0.9% (Kanner *et al*., 2001). A study done by Tesoriere *et al.* (2004) showed that 0.2 μM plasma concentration is attained after single ingestion of 500 g cactus pear fruit pulp, providing 16 mg of betanin, with bioavailability around 0.68%. The findings from both studies showed that the bioavailability of betanin is lower than 1% of the administered amount. The low bioavailability of betanin could be attributed to food source and processing, food matrix, digestive instability and mechanisms of absorption (Pavokovic and Krsnik-Rasol, 2011). Due to poor bioavailability, highly concentrated betanin is needed in single ingestion in order to attain desired concentration of betanin in body compartment.

Numerous studies have demonstrated that fermentation is an alternative promising technique to improve the bioactive compounds profile in fruits and vegetables such as cactus pear fruits (Castellar *et al.*, 2008), orange juice (Cerrillo *et al*., 2014), Malabar spinach (Kumar *et al*., 2014), red sorghum (Svensson *et al.*,2012), and onion (Yang *et al*., 2012). Recent study done by Foong *et al*. (2012) has successfully identified several bioactive compounds (campesterol, stigmasterol, B-sitosterol, betanin and isobetanin) in fermented red dragon fruit with betanin as the prominent bioactive compounds present at concentration of 14.23 g/L. Accordingly, it would be valuable to investigate the effect of different fermentation parameters on the betalains concentration in fermented red dragon fruit drink to produce drink with optimum betalains concentration (Adetuyi and Ibrahim, 2014).

Therefore, the effect of fermentation parameters, namely (i) fermentation duration (ii) white refined cane sugar to flesh ratio and (iii) types of sugar on concentration of betalainic (betanin, isobetanin) and non-betalainic phenolic compounds in fermented red dragon fruit drink were studied in this study.

Materials and Methods

Materials and chemicals

Approximately 5 kg of red dragon fruits (RDF) were purchased from a local market (Aeon, Kuala Lumpur, Malaysia). The selected fruits were all at commercial maturity level, without damage, insect and foreign matter. White refined cane sugar and brown raw cane sugar were purchased from local markets. All chemicals used were analytical grade (Fisher Chemical, UK; TCI, Japan; Merck, Germany; Millipore, USA).

Sample preparation

RDF with an average weight of 0.4-0.6 kg was rinsed with tap water to remove dirt and residues followed by air dried. Then, the skin of RDF was peeled while the flesh was cut into pieces (Foong *et al*., 2012).

Fermentation

Fermentation of RDF was carried out in 2 L stainless steel fermentation tanks (140 OD x 100 ID x 380 mm H) according to Foong *et al.* (2012) with slight modifications. Red dragon fruit were blended and poured into fermentation tanks and added with sugar in alternate layer. Then, fermentation tanks were closed tightly and stored in a clean cabinet at 25°C. The fermented drink from each tank was strained, pasteurized at 72°C for 15 s and stored in bottles at -20°C until further analysis. Several parameters were investigated following single factor experiment:

Effect of fermentation duration

Four levels of fermentation duration namely (i) 1 day (ii) 3 days (iii) 5 days (iv) 7 days (v) 9 days using 10% white refined cane sugar to flesh ratio (w/w) were carried out. The best fermentation duration was selected based on the values of betacyanins (betanin and isobetanin, g/L), TPC (mg gallic acid equivalent, GAE/100mL) and TFC (mg catechin equivalent, CE/100mL), respectively.

Effect of white refined cane sugar to flesh ratio

Three levels of white refined cane sugar to flesh ratio namely (i) 10% (ii) 15% (iii) 20% (w/w) were used in RDF fermentation. The optimum fermentation duration determined from previous section were chosen (7 days). The best white sugar to flesh ratio was selected based on the values of betacyanins (betanin and isobetanin, g/L), TPC (mg gallic acid equivalent, GAE/100mL) and TFC (mg catechin equivalent, CE/100mL), respectively.

Effect of types of sugar

Red dragon fruit was fermented with optimum fermentation duration and white refined cane sugar to flesh ratio selected previously using brown raw cane sugar (10% sugar to flesh ratio). The favorable type of sugar was selected based on the values of betacyanins (betanin and isobetanin, g/L), TPC (mg gallic acid equivalent, GAE/100mL) and TFC (mg catechin equivalent, CE/100mL).

Ethanol analysis

The determination of ethanol content was done according to Foong *et al.* (2012) with slight modifications. The alcohol content of sample was determined using Agilent A7890 gas chromatography (Agilent Ins, US), equipped with a computer containing integrator software (ChemStation), a 30m DB-Wax capillary column (250 µm id, film thickness: 0.25 µm) and a flame ionization detector (FID) (H_2: 30 mL/min and air: 400 mL/min). Sample was first diluted 5 times with ultra-pure water and filtered into each vial using nylon syringe filter of pore size 0.45 µm prior to injection into gas chromatography (GC) through automated injection method. All of the samples were assayed in triplicate.

Betacyanins analysis

Betacyanins quantification was done according to Foong *et al.* (2012) with slight modifications. An Agilent 1200 series HPLC system fitted with G1322A degasser, C1328B injector, G1329A column oven and G1315D diode array detector (DAD) was employed. The column used throughout this study was a reversed phase Zorbax Eclipse Plus C18 column 3.5 Micron (2.1 x 150 mm). Working solutions of betanin standard (TCI, Japan) which consists of betanin and isobetanin (2 g/L, 4 g/L, 6 g/L, 8 g/L and 10 g/L) was prepared. Each sample was analyzed in triplicate.

Total phenolic content

Determination of total phenolic content was done according to Foong *et al.* (2012) and Thoo *et al.* (2010) with sight modifications using Folin-Ciocalteu (FC) reagent. Briefly, 1 mL of diluted sample was mixed with 1 mL of FC reagent (10 fold dilution). After 3 mins, 800µL of sodium carbonate anhydrous solution (7.5% w/v) was added and vortexed for 10s. Subsequently, the mixture was incubated in the dark at room temperature for 2 hr followed by absorbance measurement at 765 nm against blank using UV-vis spectrophotometer (UviLine9400, France). Results were expressed as mg of gallic acid equivalent (GAE) per 100 mL of sample.

Total flavonoid content

Determination of total flavonoid content was performed according to procedures described by Foong *et al.* (2012) and Thoo *et al.* (2010) with slight modifications. Briefly, 0.25 mL of sample was added to 1.25 mL deionized water. At time zero, 75 µL of 5% sodium nitrite was added into the mixture, followed by 150 µL of 10% aluminium chloride hexahydrate 6 mins later. At 6 mins later, 0.5 mL of 1 M sodium hydroxide and deionized water was added to make up to a total volume of 2.5 mL and vortexed for 10s. Immediately, absorbance of reaction mixture was measured at 510 nm against blank. Total flavonoid content was expressed as catechin equivalent (CE) per 100 mL of sample.

pH analysis

The pH of samples were measured using pH meter (Mettler Toledo, Switzerland). Measurements were done in triplicate and average reading was obtained for each sample.

Total soluble solids (TSS)

Hand-held refractometer with detection Brix° range of 0-32 Brix° (Fisher Scientific, UK) was used to determine the TSS of samples. A few drops of sample were placed on the prism surface with daylight plate closed gently. Refractometer was held in direction of natural light and reading was recorded (Foong *et al*., 2012).

Water activity

The water activity of samples was measured at 25°C using an electronic dew-point water activity meter, AquaLab Pre (Decagon Devices, USA). For each determination, three replicates were obtained and the average readings were reported (Zamora and Chirife, 2006).

Statistical analysis

All experiment results were analyzed using Statistical Package for Social Science software (SPSS version 23.0). All numerical data were expressed as mean ± standard deviations of triplicate measurements of replicate fermentations. One-way analysis of variance (ANOVA) with Tukey's test was used to determine significant differences ($p<0.05$) between means.

Results and Discussion

Fermentation of red dragon fruit

Red dragon fruit is rich in betalains, mainly composed of the red-violet betanin and the C15-isomer isobetanin (Foong *et al.*, 2012). Fermentation techniques was adopted for betalains concentration as suggested by studies done by Castellar *et al.* (2008) and Kumar *et al.* (2014) on Opuntia fruit and Malabar spinach, respectively. Red dragon fruit

Table 1. Effect of fermentation duration on betacyanins, total phenolics content and total flavonoids content of fermented red dragon fruit drink

Fermentation duration (Days)	Betanin (g/L)	Isobetanin (g/L)	TPC (mg GAE/100mL)	TFC (mg CE/100mL)
1	24.50±1.99[d]	8.85±2.17[e]	435.11±2.89[e]	2.34±1.41[d]
3	43.30±1.83[c]	13.23±2.09[c]	536.69±3.82[d]	6.94±1.04[c]
5	82.47±2.29[b]	24.18±2.09[b]	882.40±2.74[c]	11.61±1.28[a,b]
7	112.67±3.19[a]	34.47±3.81[a]	1001.07±2.37[a]	17.66±1.32[a]
9	82.97±2.31[b]	38.70±3.16[a]	908.30±2.18[b]	14.21±1.26[a]

Data are expressed as mean ± SD values (n = 3). Mean value with different superscript in each column differs significantly ($p<0.05$).

Table 2. Effect of white refined cane sugar to flesh ratio on betacyanins, total phenolics content and total flavonoids content of fermented red dragon fruit drink at day 7

Sugar to flesh ratio (%, w/w)	Betanin (g/L)	Isobetanin (g/L)	TPC (mg GAE/100mL)	TFC (mg CE/100mL)
10	131.68±3.16[a]	39.76±2.53[a]	1136.85±3.32[a]	10.39±1.78[a,b]
15	126.76±2.84[b]	33.93±2.26[b]	1012.07±3.53[b]	10.15±1.39[b]
20	95.32±3.87[c]	23.67±3.42[c]	987.67±1.90[c]	8.00±1.28[c]

Data are expressed as mean ± SD values (n = 3). Mean value with different superscript in each column differs significantly ($p<0.05$).

is reported to contain 0.23-0.39% of betacyanins in the fruit pulps (Stintzing *et al.*, 2002). However, the fruit pulp is reported to contain 11.70 mg/mL of betacyanins, which is about 1.1% in another study (Phebe *et al.*, 2009). In present study, the betanin content in fermented red dragon fruit drink, has successfully increased through fermentation technique, highest at 131.68 g/L and lowest at 24.50 g/L, range from 2.5% to 13.1% (Table 1 and 3). It was observed that total phenolic content increased with betanin content as shown in Table 1, Table 2 and Table 4 in fermented red dragon fruit drink. It was suggested that betacyanins contain both phenolic and non-phenolic structures, hence contributing to the total phenolic content (Nurliyana *et al.*, 2010; Foong *et al.*, 2012). Nevertheless, higher amount of total phenolic content (435.11-1136.85 mg GAE/100mL) was found in fermented red dragon fruit drink compared to fruit flesh (42.4 mg GAE/100g fresh weight) which further supported that antioxidant potential was enhanced through fermentation (Wu *et al.*, 2006). This is in consistent with the findings of Dordevic *et al.* (2010), where total phenolic content of buckwheat extracts increased by 17.2% after fermentation. On the other hand, total flavonoid content present in much lower amount in fermented red dragon fruit drink (2.34-17.66 mg CE/100 mL). This could be due to inappropriate use of aluminum chloride colorimetric method in present study as this method only detect flavones and flavols that were found complex stably with aluminum chloride (Foong *et al.*, 2012). Even so, total flavonoid content recorded in present study was higher compared to fruit pulps (7.21 mg CE/100g fresh weight) too (Wu *et al.*, 2006). As a result, it was suggested that quantification of non-betalainic phenolic compounds can be used as a quality control indicator instead of betalainic phenolic compounds.

Effect of fermentation duration

The fermentation duration must be optimized to reduce run time and more critically, to increase betalain content of fermented red dragon fruit drink. It was suggested in various studies that reducing fermentation time could enhance antioxidant potential (Chang *et al.*, 2009; Adetuyi and Ibrahim, 2014). Table 1 shows that betanin and isobetanin content increased with increasing fermentation duration up to 7 days followed by a decreased at 9 days of fermentation. The betanin content has increased significantly from 24.50 g/L to 112.67 g/L, up to 78.3% increment from day 1 to day 7 fermentation. In contrast, isobetanin content has increased significantly from day 1 to day 9 fermentation,

Table 3. Effect of white refined cane sugar to flesh ratio on physicochemical properties of fermented red dragon fruit drink at day 7

Sugar to flesh ratio (%, w/w)	Ethanol (%)	pH	TSS (Brix°)	a_w
10	1.72±0.03^a	3.13±0.02^c	25.2±0.10^c	0.970±0.00^a
15	1.68±0.02^b	3.57±0.01^b	26.2±0.12^b	0.968±0.00^b
20	1.66±0.04^b	3.77±0.01^a	28.7±0.12^a	0.954±0.00^c

Data are expressed as mean ± SD values (n = 3). Mean value with different superscript in each column differs significantly (p<0.05)

Table 4. Effect of types of sugar on betacyanins, total phenolics content and total flavonoids content of fermented red dragon fruit drink at day 7

Types of sugar	Betanin (g/L)	Isobetanin (g/L)	TPC (mg GAE/100mL)	TFC (mg CE/100mL)
White refined cane sugar	85.04±2.15^a	35.91±2.03^a	1001.07±2.37^a	17.46±0.16^a
Brown raw cane sugar	74.19±1.73^b	25.23±1.27^b	920.81±1.99^b	14.21±0.24^b

Data are expressed as mean ± SD values (n = 3). Mean value with different superscript in each column differs significantly (p<0.05)

up to 77.1%, from 8.85 g/L to 38.70g/L. Betanin, phyllocactin, hylocerenin and their respective isomers were identified as the major betacyanin components in red dragon fruits with phyllocactin represented the predominant betacyanin (Naderi *et al.*, 2012). However, phyllocactin was the least stable and degraded faster compared to the higher stability betanin and isobetanin pigments (Gengatharan *et al.*, 2016a). Furthermore, pasteurization treatment after fermentation could induce phyllocactin deacylation, resulting in betanin formation in end products. This was reported in various studies whereby phyllocactin is more prone to deacylation, especially in thermally treated samples (Gengatharan *et al.*, 2016b). It was suggested that the combined effects of microbes activity and thermal exposure during pasteurization promotes isomerization of unstable betanin structure into isobetanin, hence isobetanin content was found increase across fermentation duration (Azeredo, 2009). Determination of optimum fermentation duration is crucial as further increased in fermentation duration might allow microorganisms to consume available compounds for growth, hence reducing concentration of betanin. Nevetheless, lengthened fermentation duration could also contribute further acidification as well as undesired chemical reactions between compounds leading to reduced betanin (Adetuyi and Ibrahim, 2014). As a result, the best fermentation duration for fermented red dragon fruit drink was selected to be day 7. This finding is further supported by Foong *et al.* (2012), where only 14.23 g/L betanin was found in fermented red dragon fruit drink that has been subjected to 8 weeks fermentation. Thus, present study has successfully improved betanin content at least 7 fold (112.67 g/L) by reducing fermentation duration from 8 weeks to 1 week.

Effect of white refined cane sugar to flesh ratio

In consideration of high sugar to flesh ratio will have adverse effect on activity of microorganisms, due to high osmotic pressure. This will inhibit metabolism of microorganisms and may decreased in amount of viable cells (Ozmihci and Kargi, 2007). Therefore, white refined cane sugar to flesh ratio 10-20% (w/w) were evaluated in present study. Table 2 shows that 10% sugar to flesh ratio has produced significantly higher betanin content compared to 15% (126.76 g/L) and 20% (95.32 g/L). This could be due to increased rate of microorganisms activity which indicated by significantly higher ethanol content and lower pH recorded in Table 4. Red dragon fruit is reported to contain considerably amount of protein (0.16-0.23 g/100g fruit flesh) (Khalili *et al.*, 2006), hence the breakdown of protein to free amino acids, particularly tyrosine for biosynthesis of betacyanins, by microbial protease activity could account for the increased in betanin content too (Adetuyi and Ibrahim, 2014; Esatbeyoglu *et al.*, 2015). Besides, metabolism activity of microorganisms produced proteolytic enzyme that could hydrolyze complexes of bounded phenolics into soluble free phenols, thus

Table 5. Effect of types of sugar on physicochemical properties of fermented red dragon fruit drink at day 7

Type of sugar	Ethanol (%)	pH	TSS (Brix°)	a_w
White refined cane sugar	2.11±0.06[b]	3.12±0.02[a]	28.9±0.12[b]	0.952±0.001[a]
Brown raw cane sugar	2.41±0.03[a]	2.87±0.01[b]	29.9±0.12[a]	0.950±0.001[b]

Data are expressed as mean ± SD values (n = 3). Mean value with different superscript in each column differs significantly (p<0.05).

releasing bounded form betacyanins to increase the concentration (Adetuyi and Ibrahim, 2014). Apart from microbes' activity, reverse osmosis process was applied for concentration of betacyanins too (Pap *et al*., 2004). Red dragon fruit is reported to have high moisture content, up to 87.3% (Mohd Adzim Khalili *et al*., 2006). Therefore, arrangement of sugar and blended fruit flesh in layers could aid in extraction of water soluble pigments (betacyanins), from tissue into the drink, hence concentrating betacyanins content. In addition, microbes may consume sugar for growth and this reduces the total soluble solids content thereby enhancing the release of water soluble betacyanins into the drink (Kumar *et al.,* 2014) This implicated that both microbes activity and reverse osmosis process is not optimized using 15% and 20% sugar to flesh ratio. Consequently, the selected best sugar to flesh ratio was 10%, which produced significantly higher betacyanins content. Nevertheless, the value of betanin content obtained through these two single factor (fermentation duration and sugar to flesh ratio) experiments was observed to be significantly different, even with the same fermentation parameters. This could be due to the constituents in natural fruits, including the pre-harvest and post-harvest factors even though they were bought from the same sources (Tay *et al.,* 2014).

Effect of types of sugar

White refined cane sugar was compared to brown raw cane sugar in present study as an alternative low cost raw material. Remarkably, white refined cane sugar served as a better choice compared to brown raw cane sugar with significantly higher yield of betacyanins as shown in Table 5. The difference between brown raw cane sugar and white refined cane sugar is the former contains 187 mg calcium, 56 mg phosphorus, 4.8 mg iron, 9.7 mg magnesium, 757 mg potassium and 97 mg sodium, while the latter contains scant traces of nutrients only (Demo *et al*., 2008). These minerals could act as catalyst and increased the reaction rate by lowering activation energy as indicated by the significantly higher ethanol and total soluble solid content as shown in Table 5 (low sugar consumption by microbes) (Felix *et al*., 2014). The minerals might present in minute amounts, but are essential activators and modulators of numerous biological activities for microbes' performance and survival. For instance, potassium could maintain charge homeostasis, magnesium acts as enzyme activator and calcium aids in membrane maintenance (Udeh and Kgatla, 2013). Consequently, the rate of sugar consumption by microbes decreased (higher total soluble solids content), which suppressed the release of water soluble betacyanins into the drink. Therefore, white refined cane sugar with significantly higher yield of betanin (85.04 g/L) was selected as compared to brown raw cane sugar (74.19 g/L).

Conclusion

To conclude, the experimental results revealed that fermentation duration, white refined cane sugar to flesh ratio and types of sugar had significant effects on the yield of betalainic (betanin, isobetanin) and non-betalainic phenolic compounds in fermented red dragon fruit drink. The best fermentation parameters were 7 days fermentation by using 10% (w/w) white refined cane sugar. The concentration of betanin obtained through best fermentation parameters (85.04 g/L and 131.68 g/L) in present study has increased more than five-fold compared to non-optimized fermentation (14.23 g/L). Future studies of FRDFD can be aimed at storage stability of betanin under storage conditions to enhance its potential as functional drinks.

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