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Optimization on pectinase extraction and purification by yeast fermentation of oligosaccharides from dragon fruit *(Hyloceus undatus)*

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Abstract

Dragon fruit is becoming more popular due to their nutritional benefits. It has been reported as a potential source of natural prebiotic since it contains oligosaccharides. This research aimed to optimize conditions for extraction and purification of oligosaccharides from dragon fruit's flesh. The extraction was performed using pectinase at various concentrations while the purification was carried out using yeast *(Saccharomyces cerevisiae)* fermentation. The optimal concentration of pectinase was 124 units/g solid since the oligosaccharides yield was not significantly ($p>0.05$) different compare to pectinase at concentration of 177 units/g solid. The optimal extraction conditions of dragon fruit's flesh were using pectinase of 124 units/g solid at 40°C for 45 min. The yields of oligosaccharides, sucrose, glucose and fructose and were 34.52, 2.12, 49.20 and 14.17% on dry basis, respectively. The optimum conditions for purification of the extract with *Saccharomyces cerevisiae* obtained by using 2.5% (v/v) inoculum. Addition of urea at concentration of 0.1% (w/v) in the dragon fruit's extract showed the highest on removal of sugars during fermentation by yeast. The yeast fermentation at 30°C for 96 h could completely remove glucose, fructose and sucrose moreover it did not affect the oligosaccharides content.

Introduction

Dragon fruit (pitaya) is a fruit of the cactus species, in the genus Hylocereus and Stenocereus. Generally, it comes in three types: Hylocereus polyrhizus (red pulp with magenta skin), Hylocereus megalanthus (yellow pulp with white skin), and Hylocereus undatus (white pulp with red-magenta skin)) (Ariffin *et al.*, 2008). In South East Asia, one of widely grown variety is red pitaya with white-flesh. However, other varieties including Hylocereus polyrhizus and *Hylocereus megalanthus* are also commercialized (Barbeau, 1990). Currently, there is much interest in developing this crop beyond the local Asian markets; Singapore, Hong Kong, Taiwan, Philippines, Malaysia and Thailand (Hoa *et al.*, 2006).

The dragon fruit pulp consists of highly gelatinous carbohydrate fibers (cellulose, hemicellulose) and simple saccharides, vitamin C, minerals and other forms of polysaccharides such as starch and pectin (Nur'aliaa *et al.*, 2010). Dragon fruit's flesh extract or juice is turbid, very viscous and contains colloidal suspension. It also contains non-digestible oligosaccharides known as prebiotics (Wichienchot *et al.*, 2010). It has been reported that oligosaccharides have beneficially impacted the host by stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thereby improving the host's health (Gibson and Roberfroid, 1995) and become the important ingredients in functional food. The oligosaccharides can be extracted from dragon fruit's flesh by using either alcohol or hot water (Wichienchot *et al.*, 2010; Daseasamoh *et al.*, 2012). They found these type of solvent and temperature remarkably influence oligosaccharide extraction efficiency. Alternatively, enzymatic extraction or hydrolysis is possibly an effective method to hydrolyze pectin and thus reduce its viscosity and possibly obtain high yield of oligosaccharides (Sin *et al.*, 2006).

The oligosaccharides extracted from various materials can generally be purified by chromatographic and membrane filtration techniques (Goulas *et al.*, 2002). Most highly purified products are usually obtained by chromatographic techniques. However, this technique is costly and hardly employed to produce in large scale for commercialization. Therefore, the possibility of using membrane filtration as an alternative technique has been extensively studied in the recent years. Generally, nanofiltration can be employed to separate low molecular weight solutes e.g. sugar, salt (Atra *et al.*, 2005). However,

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it has a limit in separation of disaccharides from low molecular weight oligosaccharides as indicated by their retentions (Goulas *et al*., 2002). Nobre and others (2012) have studied fructo-oligosaccharides purification with activated charcoal column that found that mixtures of sugar containing 50.6% (w/w) of FOS was purified to 92.9% (w/w) with a FOS recovery of 74.5% (w/w). Moreover, with the proposed process, fractions with purity up to 97% (w/w) of FOS were obtained. Alternatively, yeast fermentation is possibly a promising method to remove the mono- and di-saccharides from the dragon fruit flesh's extract, containing mono- and disaccharides and oligosaccharides (Wichienchot *et al.*, 2010). Thus high purity of oligosaccharides is expected to be obtained. *Saccharomyces cerevisiae* is well-known yeast which is a facultative anaerobe that is able to live on various fermentable and non-fermentable carbon sources. Wild-type *S. cerevisiae* strains readily utilize glucose, mannose and fructose via the Embden-Meyerhof pathway of glycolysis, while galactose is fermented via the Leloir pathway (van Marisand *et al*., 2006).

This study aims to optimize the process for extraction of oligosaccharides from dragon fruit's flesh using enzymatic hydrolysis by pectinase and purification of the extract using yeast fermentation method. The effect of hydrolysis conditions including pectinase concentration and hydrolysis time on the yields of oligosaccharides and other sugars were studied. In addition, the effects of yeast and nitrogen source concentration as well as fermentation time on mono-/di-saccharide removal were also carried out.

Materials and Methods

Raw materials, chemicals, media, microorganism

White-flesh of dragon fruit (~45 days) was purchased from a local plantation, Songkhla province, Thailand. The moisture, protein, fat, crude fiber, ash and carbohydrate of the sample analyzed according to A.O.A.C. (2000) were 83.05, 0.40, 1.26, 1.70, 2.20 and 11.39, respectively. The dragon fruit's flesh was blend, packed in plastic bag and kept at -20°C before use. All chemical reagents and pectinase from *Aspergillus niger* were purchased from Sigma-Aldrich Co. Ltd (Germany). Optimum working temperature and pH of pectinase are in the ranges below 50°C and 3.5-6, respectively (Kashyap *et al*., 2001). *S. cerevisiae* strain TISTR 5019 was obtained from the National Microbial Collection, National Research Council of Thailand (NRCT), Bangkok, Thailand.

Effect of pectinase concentration on sugar yield

The dragon fruit's flesh sample was peeled mixed with water at the weight ratio of flesh to water of 1: 2 (w/w). In order to select the optimum enzyme to substrate ratio, the effect of pectinase concentrations (17, 53, 88, 124 and 177 units/g solid, based on the dry basis of dragon fruit's flesh) on sugar yield was studied. The enzymatic hydrolysis was carried out in 3 L stirred reactor tank at constant temperature of 40±2°C (Nur' aliaa *et al*., 2011). Note that the initial pH of the mixture was <5.6. The sample was taken at 5 h of hydrolysis and then boiled (<100°C) for 5 min to inactivate the enzyme (Combo *et al*., 2012). And it was filtered to remove the seeds by cheesecloth. Then the contents of oligosaccharides, mono-/di-saccharides of the taken samples were analyzed by using HPLC with RI detector. The yield of each sugar was calculated according to the following equation;

Yield (%dry basis) = (final sugar, g)/(weight of dry solid, g).100

$$\frac{finalsugar}{initialsuagr} \times 100\left(\frac{initialsugar - finalsugar}{finalsugar} \times 100\right)$$

Effect of hydrolysis time on sugar yield

The optimal pectinase concentration resulting in the highest yield of oligosaccharides from the previous section was used for studying the effect of hydrolysis time on sugar yield. The samples were taken at 0, 1, 2, 3, 4 and 5 h of hydrolysis and then filtered to remove the seeds by cheesecloth. The sugar contents of the samples including mono-/di-saccharides and oligosaccharides were analyzed. The yield of each sugar in the extract was calculated.

Preparation of S. cerevisiae *TISTR 5019 and the extract of dragon fruit's flesh*

S. cerevisiae strain TISTR 5019 was used for removal of monosaccharides (glucose and fructose) and disaccharide (sucrose) in the dragon fruit' flesh extract by fermentation. The cultivation conditions for fermentation were 30°C and 2.5% yeast inoculum (18-h growth). The extract, obtained using optimal pectinase extraction condition from the previous section was chosen. The extract was subjected into a sterile glass bottle in a water bath at 80°C for 10 min then it was cooled to 30±2°C before *S. cerevisiae* TISTR 5019 was inoculated.

Effect of inoculum size of S. cerevisiae *TISTR 5019*

The extract sample (100 ml) obtained from the previous section was subjected to 250 ml of Erlenmeyer flask with aseptic technique. Then the sample in each flask was inoculated with *S.*

cerevisiae TISTR 5019 at varying concentrations of 0, 1.25, 2.5, 5 and 10% (v/v). The fermentation was carried out for 72 h on a shaker (180 rpm) after that the samples were taken and boiled for 10 min to kill the yeast, then centrifuged (10,000×g) for 10 min to remove the yeast cells. The sugar contents of the supernatants were determined. The growth of *S. cerevisiae* TISTR 5019 was determined by spread plate (A.O.A.C, 2001). The percentage of sugar removal was calculated according to the following equation;

Percentage of sugar removal (%dry basis) = (Initial sugar (g)-final sugar (g)/(initial sugar (g))×100

Effect of urea concentration on sugar fermentation

The optimal concentration of *S. cerevisiae* TISTR 5019 that gave the highest of mono- /di-saccharide removal was investigated. In this study, urea was used as a sole nitrogen source for studying its effect at varying concentrations (0.1, 0.3, 0.5, 0.7 and 1% w/v) on sugar fermentation. The effect of urea concentrations was carried out for 72 h at 30±2°C. The samples were taken every 6 h for sugar content analysis. The growth of *S. cerevisiae* TISTR 5019 was also determined.

Effect of fermentation time on sugar removal

The optimal inoculum size of *S. cerevisiae* TISTR 5019 and urea that gave the highest removal of mono-/di-saccharides were used. The fermentation times were varied up to 5 d. The samples were taken at 0, 1, 2, 3, 4 and 5 d for sugar content analysis. The growth of *S. cerevisiae* TISTR 5019 and sugar contents were determined by spectrophotometer and HPLC, respectively.

Determination of sugar content

Monosaccharide (glucose and fructose) and disaccharide (sucrose) and oligosaccharides (DP = 3-7) were analyzed using HPLC (Agilent, 1200s, Germany). The freeze-dried sample was prepared in the aqueous solution (1% w/v) and filtered through 0.2 µm filter. The injection volume was 10 µl and Rezex RNM-Carbohydrate Na+ column (300 × 7.8 mm) was used. The mobile phase was HPLC grade water and its flow rate was 0.4 ml/min. The refractive index (RI) detector was used and the operating temperature of the column was maintained at 80°C. Glucose, fructose and sucrose were used as mon-/di-saccharide standards, respectively, while maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose were used as oligosaccharides standards. Concentration of sugars in the sample was calculated by comparison with standard peak area of the respective sugar (Wichienchot *et al*., 2010).

Statistical analyses

A Complete Randomized Experimental Design (CRD) was used. Experimental values are presented as mean ± SD. All the experiments were done in triplicate. Obtained data were subjected to statistical analysis, using analysis of variance (ANOVA), the mean comparison were carried out using Duncan's Multiple Range Test (DMRT).

Results and Discussion

Effect of pectinase concentrations on sugar yield

Besides reaction pH and temperature, the rate of enzyme hydrolysis is influenced by enzyme unit to substrate ratio. Generally, the optimum condition of pectinase is temperature below 50°C and pH 3.5-6 (Kashyap *et al.*, 2001). Thus it is worthy to study the effect of pectinase concentration to substrate ratio on the yield of each sugar to achieve the optimum condition of extraction. Figure 1 shows that the yield of oligosaccharides significantly increased (1.69 to 34.52% relative total sugar) as enzyme concentration increased (0 to 124 units/g solid) ($p<0.05$). However, it remained almost constant as enzyme concentration increased from 124 to 177 units/g solid. The yield of oligosaccharides using enzyme concentration of 0, 17, 53, 88, 124 and 177 units/g solid were 1.69, 9.44, 18.01, 28.85, 34.52 and 34.81% (relative total sugar by dry basis), respectively. Low concentration of sucrose was obtained after hydrolysis by pectinase. However, the high yields of glucose and fructose were significantly ($p<0.05$) observed when concentration of pectinase increased to 124 units/g solid. These results suggest that low molecular weight sugars were easier to release or extract compared with those larger molecules. The oligosaccharides obtained from this experiment possibly came from two sources, the oligosaccharides that already exist in the dragon fruit's flesh (Wichienchot *et al*., 2010, Daseasamoh *et al*., 2012) and the result of enzymatic hydrolysis (Sabajannes *et al*., 2012). It is worthy to note that the pH of the mixture decreased from 5.6 to 4.2 after 5 h of hydrolysis. The results indicate that not only mono-/di-saccharides and oligosaccharides, but also other sugar acids were produced. Practically, pectinase is widely used in the fruit juice industries to improve juice yield, produce clear and stable juice since it has ability to hydrolyze pectin and degrade plant cell wall that help to release the components found in the cell (Lee *et al*., 2006). Regarding oligosaccharide yield, the optimal concentration of

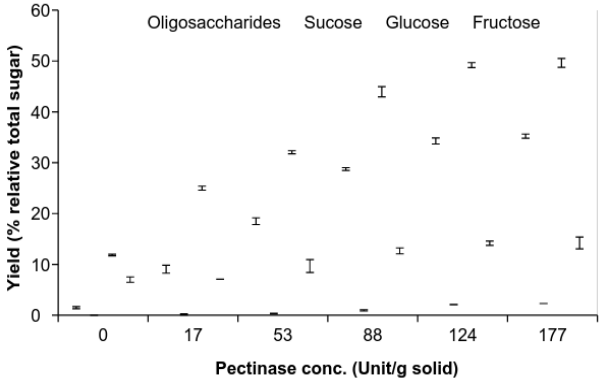

Figure 1. Yields (%) of oligosaccharides and sugars obtained after hydrolysis of dragon fruit's flesh with different pectinase concentrations (at 40°C and hydrolysis time of 5 h)

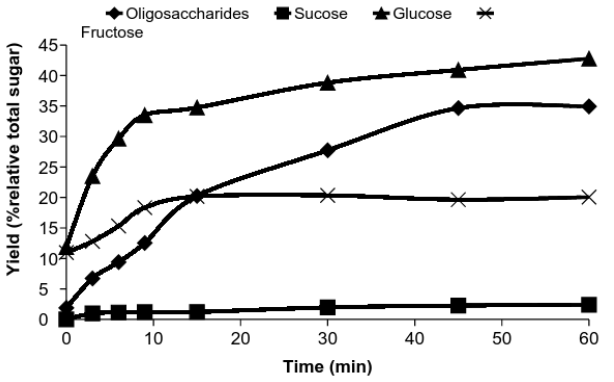

Figure 2. Yields (%) of oligosaccharides and sugars obtained after hydrolysis of dragon fruit's flesh with different hydrolysis times (at 40°C and pectinase concentration of 124 units/g solid, dry basis)

pectinase was 124 units/g solid and it was used for the next study.

Effect of extraction times on sugar yield

Extraction or hydrolysis time is also a key parameter to indicate process performance. Generally, shorter time with low enzyme concentration as well as high yield of target product with less impurity is preferred. The effect of extraction times (0, 3, 6, 9, 15, 30, 45, 60 min) using pectinase at 124 units/g solid and temperature of 40°C on the yields of oligosaccharides, sucrose, glucose and fructose is presented in Figure 2. The yields of all sugars significantly increased with extraction times ($p<0.05$) and reached the maximum yield within 60 min of hydrolysis. Thus increasing of the hydrolysis time longer than 60 min did not help to increase the oligosaccharides yield. The highest yield of oligosaccharides (34.93% (dry basis) or 7.1% (wet basis)) was obtained at hydrolysis time of 60 min. Generally, oligosaccharides extracted from plant are fructo-oligosaccharides (FOS). The FOS yields (wet basis) extracted from banana, onion and wheat were in the ranges of 0.3-0.7%, 1.1-7.5% and 0.8-4.0%, respectively (Mitsuoka *et al.*, 1987; Roberfroid *et al.*, 1993; Modler, 1994). The values are much lower compared to that obtained in this study. However, the yield of FOS extracted from dragon fruit's flesh was much lower than inulin-oligofructose found in chicory and artichoke root (18-20% wet basis) (Loo, 2006).

It is worthy to note that the highest yield was glucose (<43%) followed by oligosaccharides (<35%), fructose (<20%) and sucrose (<2.5%). Since the extract contain high content of sugar that can be either used to produce sport drink or oligosaccharides. This study is focused on prebiotic oligosaccharides that can be used as functional food supplement. Therefore, higher purity of the oligosaccharides is required to achieve high quality product. In addition, glucose, fructose and sucrose have no prebiotic property thus it should be minimized. Thus mono-/di-saccharides in the extract were further removed by yeast fermentation.

Effect of inoculum sizes of S. cerevisiae *TISTR 5019 on sugar removal*

The effect of concentrations of *S. cerevisiae* TISTR 5019 (0, 1.25, 2.50, 5 and 10%, v/v) on removal of glucose, fructose and sucrose in the extract were studied. The results showed that when the inoculum size of *S. cerevisiae* TISTR 5019 increased, the percent removal of sugar was also increased (Figure 3). The results indicate that increasing inoculum sizes of *S. cerevisiae* TISTR 5019 concentration up to 2.5% (v/v) significantly decreased the content of glucose and fructose in the extract ($p< 0.05$). However, increasing inoculum size of S. cerevisiae TISTR 5019 from 2.5 to 10% (v/v) did not have remarkably impact on glucose and fructose removal. Thus the optimal inoculum size of *S. cerevisiae* TISTR 5019 to give the maximum efficiency to remove glucose, fructose and sucrose (~60%) at 30°C for 72 h was found to be 2.5% (v/v). It is important to note that complete removal of mono-/di-saccharides is preferred. Thus factors including addition of nitrogen source (urea) and fermentation time expected to enhance such removal were studied in the next section.

Effect of urea concentrations on sugar removal

The growth of *S. cerevisiae* TISTR 5019 was possibly limited by nitrogen source, therefore, addition of urea may be required for complete fermentation. The effect on addition of urea in the dragon fruit extract at concentrations of 0.1, 0.3, 0.5, 0.7 and 1% (w/v) with *S. cerevisiae* TISTR 5019 at 2.5% (v/v) to remove glucose, fructose and sucrose in the extract for 72 h at 30°C were studied. Generally, additional urea

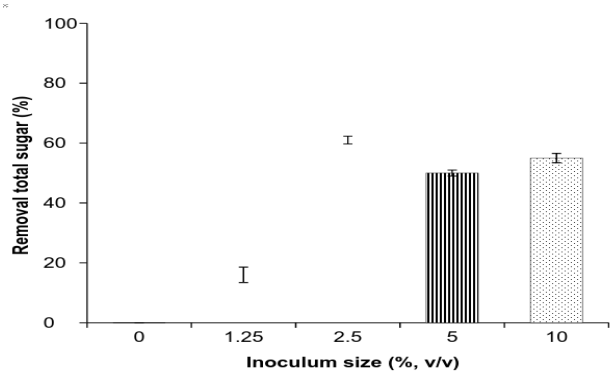

Figure 3. Effect of inoculum sizes (%, v/v) of *S. cerevisiae* on sugar removal in the extract of dragon fruit's flesh for 72 h at 30°C

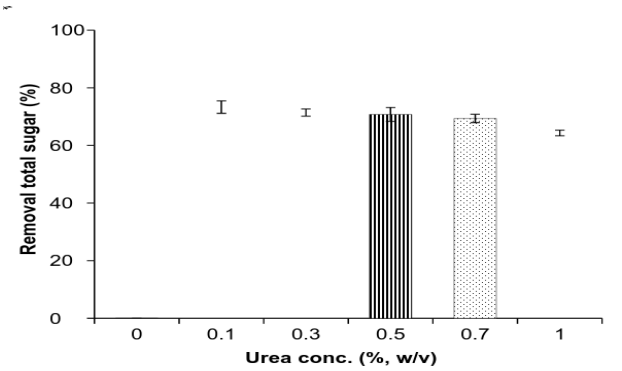

Figure 4. Effect of urea concentrations on sugar removal in the extract of dragon fruit's flesh for 72 h at 30°C

concentration increased the growth rate. However, the maximum growth rate of *S. cerevisiae* TISTR 5019 was observed when 0.3% (w/v) urea was used (Figure 4). The optimal concentration of urea which gave the maximum efficiency to remove glucose, fructose and sucrose was found to be 0.1% (w/v). At 2.5% of *S. cerevisiae* TISTR 5019 as inoculum the highest percentage on removal of sugars (glucose, fructose and sucrose) was found to be 73.31% (dry basis). Because of the amount of urea added to dragon fruit's flesh extract in fermentation with *S. cerevisiae* TISTR 5019 affected to cell growth and efficiency of sugar removal. A high concentration of urea might lead to a higher osmotic pressure to the yeast cells that suppress their growth (Bai, 2008). High osmotic pressure could also have shrunken the cells and resulted in cell dead. On the other hand, without or addition of urea at low concentration resulted in less nitrogen source to support the growth of yeast cells. The optimal concentration of urea is one of the important factor that affects cell growth and metabolizes sugars in the extract as carbon source. The mechanism of decomposition of urea into ammonia before entering into the yeast cells is carried out by urea carboxylase and allophanate hydrolase (Hofman-Bang, 1999). Zhongand *et al.* (2005) suggested that method for removal of the mono-/di-saccharide from oligosaccharide mixture by yeast must be 8-12% yeast (w/w) based on the weight of the oligosaccharide mixture and 0.1-0.5% (w/w) of carbamide as a nitrogen source. However, this study could be achieved at only 2.5% of *S. cerevisiae* TISTR 5019 due to the sugars contents in the extract was not too high (~4%). Meanwhile nitrogen source was achieved at the same concentration range at 0.1% (v/v). This study also found that yeast fermentation with or without addition of urea did not affect the oligosaccharide content of the extract.

Effect of fermentation times on sugar removal

The result of sugar content of the dragon fruit's flesh extract fermented by 2.5% (v/v) *S. cerevisiae* TISTR 5019 added with 0.1% (w/v) of urea at 30°C for 5 days (Figure 5). It was found that the free yeast cells utilized the sugars at day 1 onwards representing the lag phase of growth curve. Thereafter, glucose decreased sharply and vanished completely at day 4, whilst fructose content remains constant till day 1 and then started to decrease gradually from day 2. This suggests that free yeast cells start utilizing fructose when the glucose is exhausted from the medium. However, the free yeast cells were unable to metabolize oligosaccharides content in the extract thus no significance ($p>0.05$) reduction of the oligosaccharides was observed. Generally, yeast cells metabolize both mono-/di-saccharide simultaneously at the initial stage of fermentation (Paturau, 1989). Moreover, the results showed that glucose and fructose in the extract from dragon fruit's flesh was completely utilized at day 4 of fermentation. Whereas, sucrose was completely utilized within a short period (after day 1) of yeast fermentation. The rate of disappearance in mono-/di-saccharides compositions was ranked in the following order: sucrose>glucose>fructose within the initial 4 days of fermentation. Moreover, the sugar removal time can decrease by optimizing both of initial cell numbers of yeast and addition of nitrogen source. However, they should look at other factors such as ethanol that as a product derived from the fermentation. Yeast cells are inhibited by ethanol is in the range of 4.7 to 7.8% by weight (Bai *et al*., 2007). Furthermore, Crittenden (2002) reported that the glucose, fructose and sucrose in the extract were metabolized by yeast and then converted to ethanol and carbon dioxide. In general, ethanol and carbon dioxide are easily removed by evaporation process at elevated temperature. Hence, the fermentation of dragon fruit flesh extract using

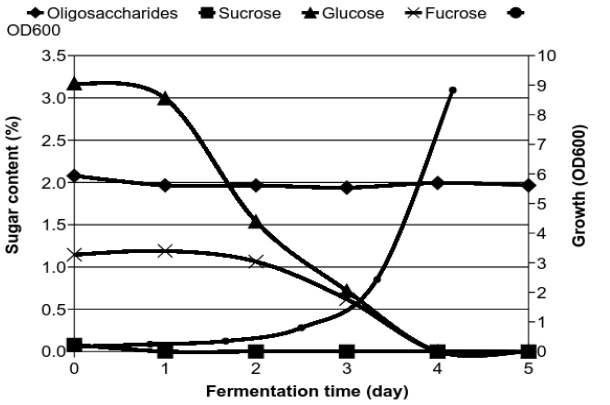

Figure 5. Effect of fermentation times of the extract of dragon fruit's flesh on sugar removal and growth of *S. cerevisiae* TISTR 5019 (2.5%, v/v), 0.1% (w/v) urea for 5 days at 30°C

yeast could serve as a promising technique in order to remove sugars, especially mon-/di-saccharide and but do not degrade the oligosaccharides in the medium. However, further improvement is needed to increase the fermentation efficiency for the application at industrial scale to make the process economical. In addition, the impurities or metabolites generated during yeast fermentation should be further recovered and utilized.

Conclusion

The optimum condition for the production of oligosaccharides of partially high purity derived from dragon fruit's flesh was successfully developed. The optimum condition using pectinase extraction was 124 units/g solid, 40°C at extraction time of 45 mins. The yield of oligosaccharides, fructose, glucose and sucrose in the extract were 34.66, 19.64, 40.91 and 2.28% (% relative total sugar in dry basis, respectively. Oligosaccharide of high purity was achieved by yeast fermentation was achieved. The optimum condition for purification of the dragon fruit extract by removal of sugars (glucose, fructose and sucrose) was achieved by using 2.5% (v/v) of S. cerevisiae TISTR 5019, with addition of 0.1% (w/v) urea and fermentation time of 4 days at 30±2°C under shaking at 180 rpm. The yeast fermentation under this optimal conditions could completely remove glucose, fructose and sucrose with no effect on oligosaccharides content.

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