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Application of membrane-based technology for purification of bromelain

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Abstract

About 60% of world's commercial enzyme products are proteases, giving promising opportunity to derive such enzymes sustainably from waste sources. Bromelain is a crude protease occurring naturally in pineapple, and it possesses properties of benefit for pharmaceutical, medical and food products. The production of bromelain involves a purification stage, normally performed by small-scale conventional operations which lead to high operating cost and low product recovery, while being difficult to scale up and produce polluting by-products. Membrane-based technology offers an alternative to produce high quality purified bromelain in a more efficient and sustainable process. This review identified the current state and future needs for utilising membrane processes for sustainable bromelain production at larger scales. It was found that declining membrane flux due to fouling have been reported, but may be effectively overcome with more appropriate (and advanced) membrane types and/or processing conditions. For example, interactions between macromolecules present in the pineapple derived bromelain mixture (particularly polysaccharides) and the membrane may cause performance limiting fouling, but can be overcome by enzymatic pre-treatment. Membrane fouling can be further reduced by the employment of ceramic membrane filters operating at optimised trans-membrane pressure, cross-flow velocity, feed pH and temperature. Two-stage ultrafiltration together with diafiltration or gas sparging was suggested as a means to reduce fouling and improve enzyme purity. Despite these promising technical findings, the review identified the need for a valid economic assessment to properly guide further work towards purifying bromelain from pineapple waste for sustainable production of commercial proteases.

Introduction

The world's production of pineapple has grown at 5.61% per annum for the past 5 years (FAOSTAT, 2015). The latest data in 2013 by Food and Agriculture Organization of United Nations (FAO) showed that the total production of pineapple around the world was above 24 million tonne making it one of the top 50 commodities of food and agriculture worldwide. About 70% of the pineapple produced is consumed fresh while the remaining 30% is processed into canned slices, chunks, crush (solid pack) and juice (Heuzé *et al*., 2015). As a result, increased production of pineapple and consequently a volume of processed products has resulted in a likewise rise in the waste generation due to selection and elimination of the components unsuitable for human consumption. This waste includes the skins (outer peels), crowns, bud ends, cores, waste from fresh trimmings, the pomace of the fruit from which the juice has been extracted, leaves and other non-fruit parts (Heuzé *et al*., 2015) which are prone to microbial spoilage and thus become an environmental issue (Ketnawa *et al*., 2012). The waste represents 49 to 58% of the total pineapple weight as reported on different cultivars (Ketnawa *et al*., 2012; Nor *et al*., 2015), thus it is estimated that the pineapple waste generated worldwide each year will be approximately 12 million tonne based on the recent report on the mass of pineapple production by FAO. Interestingly, the generated pineapple waste also has the potential to be utilized in many applications including as a source of bioactive compounds, in particular a proteolytic enzyme bromelain.

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Bromelain is a crude enzyme of pineapple that contains various closely related proteinases demonstrating antiedematous (Pavan *et al*., 2012), anti-inflammatory (Oh-ishi *et al*., 1979) and antithrombotic (Bhui *et al*., 2009) properties. It can also be a platelet aggregator (Heinicke *et al*., 1972; Morita *et al*., 1979) and has potential as an anticancer agent (Chobotova *et al*., 2010). The health benefits of bromelain also include its use to relieve mild discomfort related to osteoarthritis, and as an antibiotic since it can modify the permeability of organs and tissues to different drugs (Pavan *et al*., 2012). The enzyme can also be administered to prevent diarrhoea, reduce sinus discomfort, eliminate burn debris and accelerate healing process, treating indigestion and heartburn, help balance the acidity of the stomach and boost overall immunity (Group, 2014; Novaes *et al*., 2016). Besides, it is used in the food industry as a meat tenderiser and dietary supplement (Maurer, 2001) as well as in the cosmetic industry to treat acne and wrinkles (Arshad *et al*., 2014).

Commercially available bromelain (EC 3.4.22.32) is often made from pineapple stems, even though other parts of the pineapple have also been reported to contain certain amounts of bromelain, with the crown part having the highest enzyme activity compared to other parts such as the peel and core (Ketnawa *et al*., 2012). The production of bromelain from the pineapple parts generally consists of several processes including extraction, recovery, purification and drying prior to packing before being sold at different purity levels, ranging typically from 600 to 2400 GDU/gram (Xian Lukee, 2015). The purification process determines the purity of the enzyme and affects the overall processing feasibility and efficiency and is therefore one of the potential areas for improvement in bromelain production. The conventional purification process is performed by complex, high energy and/or chemically intensive processes such as chromatography, salt and solvent precipitation and electrophoresis, that in general have inherent disadvantages such as scale-up problems, excessive costs when operated at industrial scale, low product recovery and associated environmental problems (Chaurasiya and Hebbar, 2013). The use of membrane filtration as an alternative method can provide a solution to some of these issues while producing high quality purified bromelain.

Despite its common application for concentration purpose, membrane separation processing, in particular microfiltration (MF) and ultrafiltration (UF), can also potentially be used for purifying enzymatic solutions, including bromelain, since it is a simple size exclusion system which is useful for the initial stage of isolation and purification of the enzyme. It possesses advantages in terms of practicality, minimal operating cost and is easier to scale up with high throughput and environmentally friendly (Girard and Fukumoto, 2000; Hinkova *et al*., 2002; Nor *et al*., 2016).

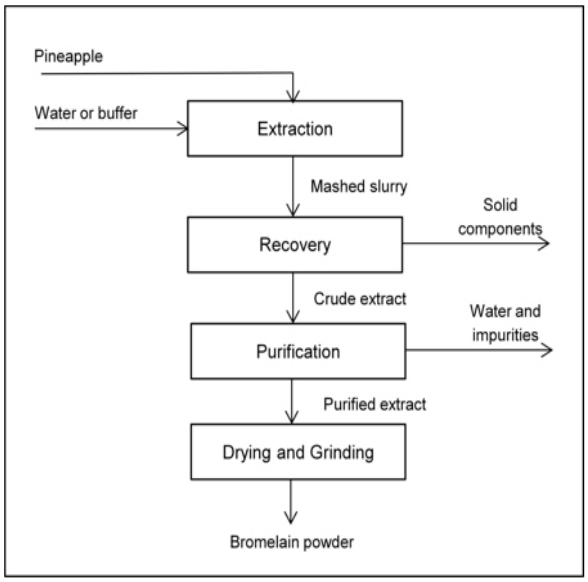

Figure 1. Flow diagram of a typical bromelain production

Despite their wide uptake in many foods, dairy and water industries, limitations related to flux decline and fouling formation require an assessment of the viability in order to adopt this technology in any specific process. It is essential to further understand flux behaviour, fouling development as well as the mechanism behind the protein separation in order to surpass these limitations, specifically in the bromelain purification application. This paper presents a review of the bromelain production and evaluates the applications of membrane technology during its purification process.

The bromelain production

Protease enzymes including bromelain dominate the enzyme market since they represent 60% of all commercialized enzymes worldwide (Feijoo-Siota and Villa, 2010). Although enzymes of microbial origin are more popular in industrial applications, the biotechnology and pharmaceutical industries are now focussing their attention on plant-based enzymes not only because of their proteolytic activity on a wide variety of proteins but also because often they are active over a wide range of temperatures and pH (Dubey *et al*., 2007; Feijoo-Siota and Villa, 2010). Between all types of plant proteases, bromelain has drawn attention in various industrial applications

due to its unique properties and higher commercial values. However, the separation and isolation of bromelain from pineapple are currently in the development stage with further exploration required to resolve challenges related to the technology, purity and economic aspects of large-scale bromelain production (Arshad *et al*., 2014).

In general, the bromelain production involves several processing steps as illustrated in Figure 1 as described by Xian Lukee (2015). The steps include: (i) extraction, (ii) recovery, (iii) purification and (iv) drying and grinding, in order to produce bromelain powder for commercialization purpose.

In the extraction step, pineapple parts are washed, cut into small pieces and undergo mechanical lysis by simply applying mechanical shear force to disrupt the plant cells and separate the enzyme from the cells by solubilizing the enzyme in water or buffer (Devakate *et al*., 2009; Lopes *et al*., 2009; Hebbar *et al*., 2012). This mechanical disruption approach is simple and suitable for pineapple cells due to their rigid structure (Illanes, 2008). However, the former method generates heat following processing that leads to enzyme denaturation, suggesting the process should be performed at low temperatures (Ahmed, 2005). Various buffers can be used during the extraction process with sodium phosphate buffer reported to yield the highest specific activity compared to potassium phosphate buffer, sodium–potassium phosphate buffer, potassium-hydroxide buffer and citrate–phosphate buffer (Chaurasiya and Hebbar, 2013).

Once the enzyme has been extracted, it is recovered in the enzyme recovery step, which implies its separation from the coarse impurities including fibres, undisrupted cells and cell debris. A solid-liquid separation process is commonly used where mashed pineapple slurry from the extraction process is filtered and centrifuged to recover the enzyme and eliminate all solid components.

Next, the crude extract is subjected to various purification techniques to remove contaminants that can interfere with bromelain intended use, as well as to increase the specific activity of the enzyme. Generally, any method suitable for protein fractionation can be used to purify bromelain. However, for production purpose, the selected purification methods are restricted to those amenable for scale-up at a reasonable cost (Illanes, 2008). Some precaution steps must be taken throughout the purification process to minimize bromelain denaturation, modification and degradation and to maximise yield (Jervis and Pierpoint, 1989).

After the purification of enzymes to the desired level, the preparations need to be formulated in accordance to their intended applications. This is an important step especially for the enzyme industry since it confers a producer the competitive edge while complying with the stringent regulations (Illanes, 2008). Most of the time, the purified bromelain is dried by spray (Cabral *et al*., 2009; Devakate *et al*., 2009) or freeze drying (Doko *et al*., 1991; Devakate *et al*., 2009) and grinded into powder. This process is performed to enhance the stability since the enzyme is unstable and has inactivation limitation in a dilute form. It is crucial to deliver bromelain in a concentrated or dried form since it maximizes its proteolytic activity *in vivo* and ensures prolonged storage stability after packaging.

Among all the various units of operation in the process of bromelain manufacture, the purification stage has a crucial role since it governs the quality of the produced enzyme in terms of purity. Besides, it is needed for concentration enrichment, removal of specific impurities and enhancing enzyme stability (Saxena *et al*., 2009) which also affect the end-product quality.

Purification process of bromelain

Apart from determining the specific activity of the enzyme, selection of a purification method significantly influences the cost of the enzyme production since the downstream processing may account ca. 60-90% of the total enzyme production cost (Lightfoot, 1990). Investigations on the efficiency of the purification process are required to reduce the cost and the purification steps, minimize the degradation of enzyme while maximising its yield. The selection of the purification method of bromelain depends primarily on the intended usage such as research, industrial, therapeutic etc. (Lopes *et al*., 2012) since each application require different enzyme purity. Bromelain from crude pineapple extract can be purified using different techniques such as by ion-exchange chromatography (Ako *et al*., 1981; Devakate *et al*., 2009), reverse micellar system (Hebbar *et al*., 2008; Hebbar *et al*., 2012), membrane filtration (Doko *et al*., 1991; Lopes *et al*., 2009; Hebbar *et al*., 2012; Lopes *et al*., 2012), gel filtration (Murachi *et al*., 1964), ammonium sulphate fractionation (Devakate *et al*., 2009), aqueous two-phase system (Babu *et al*., 2008; Ferreira *et al*., 2011) and metal affinity membranes (Nie *et al*., 2008; Zhang *et al*., 2010). Several reviews on the various bromelain purification techniques and strategies are available, including those by Nadzirah *et al*. (2013) and Arshad *et al*. (2014).

In term of purification efficiency, ion exchange

Table 1. Summarised investigations on bromelain separation by membrane filtration process

Approach	Sample	Pre-treatment	Membrane details			Processing conditions						Purification fold (by membrane process itself)	Enzyme yield (%)	References
			Membrane system	Filter material	Pore size	Surface area (m^2)	pH	TMP (bar)	Temp. (°C)	Flow rate (ml/min)	VRF			
Two-stage membrane filtration, starting by MF followed by UF	Pineapple pulp extract in phosphate buffer	-	MF - Flat sheet	Polyvinyl fluorite	0.1 µm	0.0225	7.0 & 7.5	0.05-0.15	Room temp.	-	-	-	85	Lopes *et al.* (2009); Lopes *et al.* (2012)
			UF - Centrifugal filter	-	10 kDa	-	7.0 & 7.5	-	4	-	10	-	100	
Two-stage UF system	Pineapple waste extract	-	UF stage 1 - Tubular	Ceramic (zirconium oxide)	75 kDa	0.0055	4, 5.5, 7 & 8.5	2	10 to 40	690	15	-	96.8	Nor *et al.* (2016)
			UF stage 2 - Tubular	Ceramic (zirconium oxide)	10 kDa	0.0055	4, 5.5, 7 & 8.5	2	10 to 40	690	15	2.5	-	
Purification by multiple processing steps involving MF, UF, ammonium sulphate extraction, ultracentrifugation and freeze drying	Pineapple fruit extract	12.5 ppm antifoaming agent and 200 ppm hemicellulase	MF - Tubular	Ceramic (zirconium oxide)	8 µm	0.2	8.5	-	30 ±2	-	-	-	-	Doko *et al.* (1991)
			UF - Tubular	Polysulfone	10 kDa	0.46	8.5	3	30 ±2	-	7.4-17.1	-	97	
Integration of the purification technique by coupling reverse micellar extraction (RME) process with UF	Pineapple core extract in sodium phosphate buffer	-	Tangential flow filtration (TFF)	Cellulose acetate	5 kDa	0.005	-	1	25 ±2	3	5	1.5	92.4	Hebbar *et al.* (2012)
Three-stage membrane filtration involving 2 MF processes and an UF	Pineapple waste (peel and core) extract	-	MF	-	1.2 & 0.2 µm	-	-	-	-	-	-	-	-	Gimeno *et al.* (2010)
			UF - Centrifugal filter	Regenerated cellulose	10 kDa	0.00076	-	-	-	-	-	-	-	
Purification and concentration process involving the aqueous two phase system (ATPS) , UF and direct osmosis (DO)	Pineapple core and peel extract in sodium phosphate buffer	-	UF - Flat sheet	Polysulfone	10 kDa	0.0016	7	1 to 4	25 ±2	-	-	1.2	-	Babu (2008)
			DO - Flat sheet	Hydrophilic direct osmosis	-	0.012	-	-	25 ±2	100	-	-	-	
Integration of the purification process by coupling nano-TiO_2 absorption with two-stage UF	Pineapple stem extract	-	-	-	50 and 10 kDa	-	-	2.5 - 3	-	-	-	1.4	64.7	Chao *et al.* (2009)

chromatography is one of the best purification techniques in terms of the purity increment (Nadzirah *et al*., 2013). However, some additional steps such as centrifugation, ultrafiltration and precipitation are needed together with the chromatographic method to avoid unnecessary protein binding to the column that can result in high production cost which can be a significant drawback in the large-scale downstream processing (Arshad *et al*., 2014). Other purification techniques reported also have several disadvantages such as waste disposal issue in the precipitation technique and difficult recovery of targeted protein from either the phase-forming polymer or the surfactant-containing solvent in the aqueous two phase and reverse micelle system (Jervis and Pierpoint, 1989; Arshad *et al*., 2014).

For a large-scale bromelain production, it is essential to select a purification technique that is easy to scale up with a high-throughput, environmental-friendly and being practical. The use of membrane-based processes for bromelain purification is a promising alternative since this green technology can fulfil these criteria with minimal production cost. This technology has recently gained attention particularly in the biotechnology area due to its capability of separating size and/or charge based protein with high purity and throughput (Saxena *et al*., 2009). A study on the reduction of bromelain production cost by introducing membrane processes has reported substantial cost reduction of 6.5 to 8.5 times lower than the bromelain produced by liquid-liquid extraction (Lopes *et al*., 2012). Although some limitations have been reported particularly on the declining flux and fouling formation especially when the membrane process was applied for protein separation, this can be overcome by advances in the membrane technology.

Application of membrane technology in bromelain purification

In the literature, several studies have focussed on membrane-based process for bromelain production either as a single process or in combination with other purification techniques. A multi-stage membrane process for separation and concentration of bromelain from different pineapple's parts extract has been described by Lopes *et al*. (2009), Gimeno *et al*. (2010) and Nor *et al*. (2016) which involves the microfiltration (MF) and ultrafiltration (UF) processes. Several studies on the integration of membrane process with other purification technology have been reported such as with ammonium sulphate extraction (Doko *et al*., 1991), reverse micellar system (Hebbar *et al*., 2012), aqueous two phase system (Babu, 2008) and nano-TiO_2 absorption (Chao *et al*., 2009).

Studies on utilising membranes within purifying bromelain are summarized in Table 1. Despite this promising laboratory work, the potential to implement the outcomes to large-scale bromelain production still need to be explored. The following sections discuss some important factors that need to be considered when evaluating the potential of applying the membrane processing for bromelain purification

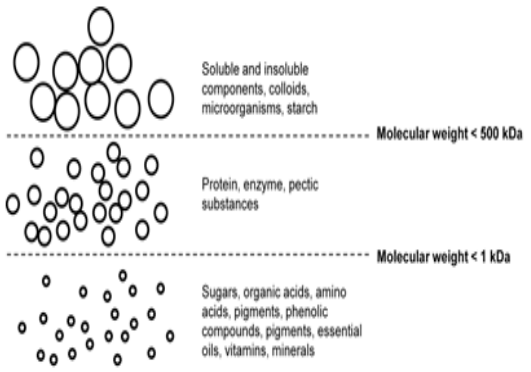

Figure 2. Typical compositions in plant extract and their corresponding molecular weights

particularly in relation to protein separation and juice clarification.

Pre-treatment considerations

The membrane processing efficiency, particularly associated to the permeate flux and membrane fouling behaviour, are strongly related to the composition of the feed. The extract from plant including pineapple may contain various components such as polysaccharides, fibres, proteins, pigments and minerals. The composition is influenced by various factors including the type of the plant, varietal characteristics, maturity, natural variation, climate and cultural practice (Girard and Fukumoto, 2000).

Figure 2 illustrates various compositions and their respective size groups typically existing in a plant extract based on Girard and Fukumoto (2000). Very large components in the plant extract typically consist of soluble and insoluble complexes, colloids (mainly polysaccharide complexes), microorganisms and starch. Proteins including enzymes and pectin are the medium size entities while small compounds in the plant extract typically include numerous sugars, organic acids, amino acids, phenolic compounds, pigments, essential oils, vitamins and minerals.

The targeted enzyme can be separated from the other components based on size exclusion. Very large particulates can easily be discarded by using centrifugation and MF process whereas nanofiltration (NF) or small molecular weight cut off (MWCO) UF can be used to filter the small size components from the targeted enzyme. Nevertheless, the separation between enzyme and protein from the pectin component is complicated and requires a precise separation based on their size. Bromelain enzyme has molecular weight (MW) of ca. 23.4 to 35.73 kDa (Arshad *et al*., 2014) while pectin has MW of 10-500 kDa with a global weight average of about 100 kDa (Girard and Fukumoto, 2000). Pectin is found to be responsible for the fouling build-up during membrane processing by forming high molecular weight aggregates which in turn hinders the membrane performance (Nor *et al*., 2015). It can be perceived as impediment to the process although it can also be considered as valuable product for some type of juice fruit processing. In the case of pineapple, it was reported that this fruit contain small but noticeable yield of pectin (Mohamed and Hasan, 1995; Normah and Ku Hasnah, 2000). Beside pectin, Grassin and Fauquembergue (1996) reported a high hemicelluloses content type galactomannans, arabinogalactans and galactoglucomannans and a natural gum (a neutral polysaccharide containing 70% sugars which are predominantly galactomannans (2.25 mannose : l galactose) which can also cause quick reduction in the ultrafiltration flux rate. Also, 0.06% (w/v) crude protein was reported in the crude pineapple waste mixture extract (Nor *et al*., 2015).

In order to reduce the potential of larger molecules to cause fouling during the membrane processing, pre-treatment of the feed should be considered. The treatment may consist of hydrolyzing soluble polysaccharides, which are responsible for high viscosity of the juice, as well as liquefying the non-soluble polysaccharides such as non-soluble pectins, cellulose, hemicellulose and lignin from cell walls (Tochi *et al*., 2009) which includes the enzymatic treatment of the feed with pectinase and cellulase enzyme.

In bromelain purification particularly, the enzyme pre-treatment has been performed by Doko *et al*. (1991) using 200 ppm hemicellulose at pH 8.5 on pineapple juice in order to reduce the viscosity and improve the filtration (Table 1). They reported that ca. 12.5 ppm anti-foaming agent was used to prevent protein loss and to stabilize the filtration rate. Moreover, there are many successful reports on pineapple juice clarification by membrane processing using the same approach. Vaillant *et al*. (2001) found that the enzyme treatment carried out before membrane filtration including for pineapple juice clarification has the advantage of lowering the juice viscosity and reducing the soluble solid content for better filtration performance. This enzymatic preparation used should contain at least sufficient pectinolytic and cellulolytic activities. Carvalho *et al*. (2008) has carried out a study on enzyme treatment on pineapple juice using commercial pectinase (Ultrazym 100G) individually, and combined with cellulase (Celluclast) in order to minimise fouling and reduce juice viscosity during the membrane process. Based on their observation, they found that the best response in pineapple juice apparent viscosity reduction (27.62%) was measured when

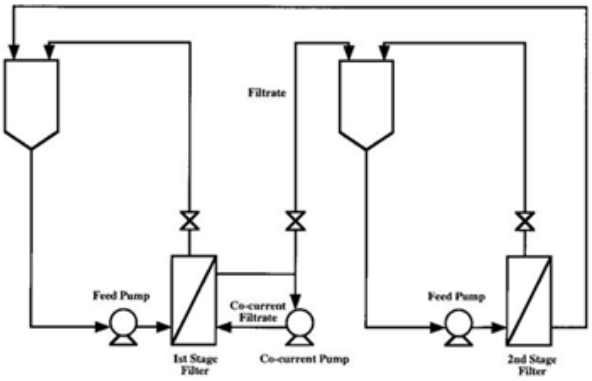

Figure 3. A two-stage UF closed-loop cascade system for protein separations using HPTFF (van Reis *et al*., 1997).

Ultrazym 100G (100 ppm) was used alone during 30 min of incubation time which subsequently led to the improvement of flux behaviour during the membrane process. Thus, it is a good practice to perform enzyme pre-treatment in order to hydrolyze pectin and other macromolecules that may interfere during the membrane processing. However, consideration on the additional production cost, processing steps and duration besides environmental impact etc. needs to be weighed up for a practical validation of this enzymatic pre-treatment.

Operation set up considerations

It is important to select a suitable membrane configuration and features for higher resolution of bromelain separation from the pineapple extract. A multi-stage membrane processing has been reported in the isolation of bromelain with or without the combination of other purification technology. It mostly involves the screening of bromelain from macromolecules during the initial filtration stage (MF) and concentration of the enzyme in the final filtration stage (UF) (Doko *et al*., 1991; Lopes *et al*., 2009; Gimeno *et al*., 2010). A further concentration step has been used by Babu (2008) using direct osmosis (DO) system to prepare the bromelain concentrate for drying.

The successful application of the multi-stage membrane system in isolating the protein of interest has been reported by many studies. This includes the usage of high-performance tangential flow filtration, known as HPTFF introduced by van Reis *et al*. (1997) which employed varies strategies by exploiting differences in both, size and charge, to achieve high resolution of protein selectivity. By performing the HPTFF system, particularly by a two-stage UF process using membrane filters with MWCO of 150 kDa and 10 kDa, they managed to separate bovine serum albumin (BSA) monomer from oligomers with 9 purification factor and 86% yield. The two-stage UF strategy compromises two UF membrane filters with the pore size larger (stage 1) and smaller (stage 2) than the targeted protein of interest as in Figure 3.

The application of the two-stage UF strategy has also been reported on different type of proteins such as to separate α-lactalbumin and β-lactoglobulin from whey protein isolate (Cheang and Zydney, 2004), ovalbumin from chicken egg white (Datta *et al*., 2009) and surfactin from fermentation broth (Isa *et al*., 2007) and can potentially be used for bromelain separation. In addition, Nor *et al*. (2016) reported an increment of 2.5-fold purity of bromelain by applying the two-stage UF system which consisted of membranes with MWCO bigger (in the UF stage 1) and smaller (in the UF stage 2) from the bromelain molecular weight. Since bromelain has a molecular weight ranging from 23.4 to 35.73 kDa (Arshad *et al*., 2014), for the two-stage UF application, the MWCO of the first stage membrane filter should be at least two times higher (75 kDa) and the second stage membrane filter two times lower (10 kDa) than that range (Nor *et al*., 2016).

The choice of membrane material in the membrane processing depends on its pressure, temperature and pH resistances and chemical compatibility (Girard and Fukumoto, 2000). Membrane process for bromelain purification requires mild processing conditions such as 0.5 to 4 bar, 10 to 30°C, pH 4 to 8.5 with no corrosive compounds or chemicals involved, which is suitable with many membrane materials. However, the cleaning procedure for the membrane is normally performed under more aggressive conditions with the usage of strong acids or alkaline detergents at a higher temperature (60-80°C). Thus, the selection of membrane materials which can withstand these extreme conditions is required.

Most of the previous studies have used polymeric membrane such as polysulfone, polyvinyl fluorite and cellulose acetate (see Table 1) for the bromelain concentration and purification process except for Doko *et al*. (1991) and Nor *et al*. (2016) who have used ceramic membranes (zirconium oxide). Ceramic membranes are classically more durable than the polymeric membranes. Examples of ceramic membranes include alumina (α-Al_2O_3 and β-Al_2O_3), zirconia (ZrO_2), titania (TiO_2), glass (SiO_2) and silicon carbide (SiC) (Lee *et al*., 2015). They exhibit far superior mechanical, thermal, chemical resistivity allowing much more extreme cleaning approaches without risk of damaging the membranes (Lee *et al*., 2013). Furthermore, ceramic membranes have been reported to have a relatively narrow pore size distribution and higher porosity (Lee *et al*., 2002; Hofs *et al*., 2011) which is suitable for bromelain

purification since better protein separation can be achieved using membrane with a smaller pore-size distribution (van Reis *et al*., 1997). Moreover, ceramic membranes exhibit lower organic fouling tendency in certain circumstances compared with the polymeric membranes due to their hydrophilic and inorganic characters (Lee and Kim, 2014). Therefore, the application of ceramic membranes for bromelain separation through membrane filtration is recommended.

Furthermore, there are operational variants for improving the membrane process for bromelain separation by introducing different approaches and techniques such as diafiltration mode, gas sparging and static mixer. These approaches have been reported to improve the protein separation while improving the filtration rate (Doko *et al*., 1991; Li *et al*., 2009, 2008). Diafiltration is the convective elimination, either continuous or discontinuous, of permeable solutes by the addition of fresh solvent to the retentate (Cheryan, 1986). A continuous diafiltration mode has been carried out by Doko *et al*. (1991) on both MF and UF to facilitate dilution and low molecular size draining through the membranes for bromelain purification. It has found to decreased total solids in the extracts and enabled better recovery of protein concentrates with high purity and concentration. The same result has been reported by Li *et al*. (2009) using diafiltration mode to separate proteases from yellowfin tuna spleen by ultrafiltration. They reported that the purity of the proteases has increased more than 10 times with better flux values in comparison to the total recycle mode. Gas sparging can be applied to improve the hydrodynamic mixing near the membrane surface and reduce concentration polarization phenomenon. The utilization of a low gas injection has been reported by Li *et al*. (2008) in order to improve critical and limiting flux during the ultrafiltration process to separate protease from yellowfin tuna spleen. On the other hand, the use of a static mixer inside a 5 nm ceramic membrane during the ultrafiltration of endo-pectinase solution improved 45% of the flux performance, 96% of the enzyme rejection, 40% of the energy saving as well as reduced 25% of the operation time (Krstić *et al*., 2007). Other advanced UF techniques for low membrane fouling, high selectivity and permeate flux in protein separation such as by using charged membranes, electro-ultrafiltration and ultrasonic UF have been extensively reviewed by (Saxena *et al*., 2009). By adopting some of these techniques in the membrane purification of bromelain, improvement on the enzyme purity and filtration rate is expected.

Processing parameters considerations

The filtration rate of the membrane process can be affected by several processing parameters such as feed pH and concentration, trans-membrane pressure, temperature, flow rate and cross-flow velocity. Table 1 includes different processing parameters reported by various studies for the membrane process of bromelain. One of the main processing factors found to be effective in increasing the flux rate is the feed pH. Lopes *et al*. (2009) investigated the influence of feed pH on the bromelain activity recovery by MF. They concluded that the best recovery of activity can be obtained at pH 7.0. The same feed pH has been selected by Babu (2008) while Doko *et al*. (1991) choose to adjust the feed pH to 8.5 in their membrane-based process for bromelain separation. To explore the role of pH more closely, Nor *et al*. (2016) investigated the effect of adjusting the feed pH on the flux behaviour, enzyme recovery and enzyme purity during the bromelain purification process. The pineapple extract used in their study was labelled as the crude waste mixture (CWM) which consisted of a specific ratio of different parts of pineapple waste including crown, peel and core. The CWM extract has been adjusted to four pH levels of 4, 5.5, 7 and 8.5. They found that a better flux behaviour can be achieved at feed pH 7 where it might be strongly related to viscosity reduction of the feed after the pH was changed away from its isoelectric point (pI) of pH 2.37 and thus, improving the filtration rate in the membrane process. This is in agreement with their previous study (Nor *et al*., 2015), where observation on the effect of the CWM extract rheological properties at different pH also found the viscosity reduction when the pH was adjusted above its pI since it lead to repulsion between the macromolecules.

Besides affecting the viscosity profiles of the feed, pH adjustment would also affect electrical charge on both, the protein and the membrane, due to the ionization or deionization of various acidic/basic groups on the protein and membrane surface (Burns and Zydney, 1999). Either attractive or repulsive response in the protein-membrane interaction might occur based on their pI. Different membrane materials have different pI such as for ceramic membranes; Al_2O_3 (pH 8-9.4), TiO_2 (pH 5.1-6.4), ZrO_2 (pH 6.3-7.1), SiC (pH 2.5-3.5), while polymeric membrane having pI of pH 4-5 (Hofs *et al*., 2011). Nor *et al*. (2015) suggested that greater feed-membrane electrostatic interactions during the membrane processing is expected if the pH of the feed is adjusted above its pI and below the membrane's pI resulting in a desired flux. It is recommended to adjust the feed to pH 6-7 if the membrane process was to be performed

using a ceramic membrane particularly if using the Al_2O_3 or ZrO_2 membrane.

The trans-membrane pressure (TMP) is also a primary processing parameter that is effective in increasing the filtration rate. In general, higher TMP leads to a higher filtration rate particularly in the membrane pressure-dependent region. However, the flux becomes independent of pressure due to the concentration polarization layer reaching its limiting concentration as the pressure increases further (Cheryan, 1986). Babu (2008) evaluated the effect of different TMP during the UF process for bromelain purification by using 10 kDa flat sheet polysulfone membrane fitted in a stirred cell by varying the TMP from 1 to 4 bar with constant stirring at 300 ppm and at room temperature of 25°C. They found that the observed permeate flux profiles were similar at all TMP with a rapid decrease in the initial stage before it stabilized due to the formation of concentration polarization layer. The average flux was found to be higher at 4 bar which was twice as the average flux value at 1 bar.

Although high TMP would lead to high filtration rate, the impact on enzyme activity should be considered. Lopes *et al*. (2009) acknowledged the loss of bromelain activity when the membrane process was performed at high TMP. The enzyme inactivation may happened by the rupture or modification of its structure while passing through the membrane pores or due to contact with fouling material. Further investigation can be performed in order to find the optimal TMP for better filtration rate without affecting the enzyme of interest.

Table 1 shows the effects of high cross-flow rate and velocity parameters on bromelain separation. Flux normally increases with improvement in hydrodynamic conditions on the surface of membrane. This is because high shear rates generated on the surface of membrane tend to shear off deposited material and consequently reduce the hydraulic resistance of the fouling layer (Cheryan, 1986). Li *et al*. (2008) observed the critical flux, limiting flux and protease selectivity at cross-flow rate of 18, 35, 52 and 70 L/h in UF process of yellowfin tuna spleen. They found that by increasing the cross-flow rate it may enhance the flux values, both for critical flux and limiting flux since it would enhance wall shear stress on membrane surface and thus reduce concentration polarization layer and external fouling. The selectivity of the enzyme has been enhanced by increasing the cross-flow rate since soluble protein and peptide transmission was promoted. Datta *et al*. (2009) studied the effects of stirring speed on the separation of ovalbumin (OVA) from chicken egg white using a two-stage UF 50 and 30 kDa stirred cell. In both UF stages, the permeate flux was found to increase with enhanced rate of stirring because of enhanced turbulence at the membrane surface, resulting in a reduction of concentration polarization. The OVA recovery increased from 82.3 to 98.7% by increasing stirring speed possibly due to lower OVA rejection on membrane surface. Nevertheless, similar situation like the TMP, vigorous hydrodynamic conditions inside the membrane might affect the enzyme structure and cause enzyme loss. Meireles *et al*. (1991) reported the increase of BSA protein denaturation rate with cross-flow velocity in the UF process. Accordingly, optimization of the related hydrodynamic conditions should be considered.

Based on the data in Table 1, most of the studies have performed the bromelain separation by membrane filtration at room temperature (~25-30°C). Increasing the processing temperatures may improve the permeation rate since at higher temperature, the membrane permeability coefficient and the diffusivity coefficient are higher, and the viscosity coefficient decreases (Girard and Fukumoto, 2000). This has been proven by Nor *et al*. (2016) who observed the effect of different processing temperatures, ranging from 10 to 40 °C, on the flux during the separation of bromelain by UF. The best flux was obtained at 40 °C indicating the need to operate the process at the highest possible temperature for the maximum flux.

However, the stability of the enzyme during the operation needs to be considered since high temperature may lead to the denaturation of protein and the reduction of bromelain activity as well as potential fouling issue by particle-particle interactions (Nor *et al*., 2015). Bromelain activity has been found to reduce by 17 % when exposed to 50°C for 60 min (Jutamongkon and Charoenrein, 2010), nevertheless it remains relatively stable at least for 1 week at room temperature (Hale *et al*., 2005). Furthermore, an increase of 3 to 4% on the average processing capacity has been reported for every 1°C increase in operating temperature between 20 and 60°C (Girard and Fukumoto, 2000) signifying the importance of ensuring optimum balance between the membrane filtration rate, enzyme stability and operating costs. It is suggested to perform the membrane purification process at a room temperature for a balance condition in regards to these factors.

Enzyme purity considerations

The purity of the separated bromelain after the membrane process is the main consideration in evaluating the performance of the membrane filtration. In general, purification fold of 1.2 to 2.5

has been reported (Table 1) by using the membrane process itself and the purity can be further improved by increasing the volume reduction factor (VRF) of the process (Nor *et al*., 2016). Besides increasing the VRF, it is believed that the bromelain purity can be further increased by implementing different filtration strategies such as using the HPTFF method as discussed in the previous section. By using this method, van Reis *et al*. (1999) reported 900-fold purification with 90% yield when separating BSA from an antigen binding fragment of monoclonal antibody (Fab). A similar work except by using a complex multi-component feed stream i.e whey protein isolate has demonstrated greater than 10-fold with 90% yield on the recovery of α–lactalbumin (Cheang and Zydney, 2004). Therefore, potential on increasing the bromelain purity by following the similar method should be considered.

Moreover, for higher grade enzyme purity, the combination of the membrane technology with other purification methods can be considered. By performing UF process after reverse micellar system in an integration purification process has resulted an increase of bromelain purity from 5.9-fold to 8.9-fold after the process (Hebbar *et al*., 2012). Purification of 1.5-fold can be achieved during the ultrafiltration process itself after 5-fold of volume reduction. In another studies, the application of membrane process with the combination of other purification methods have been found to exhibit varies level of bromelain purity including an increase of 2.0 to 2.8-fold with the combination of ammonium sulphate extraction (Doko *et al*., 1991) and 5.29-fold when coupled with the nano-TiO_2 absorption process (Chao *et al*., 2009).

Nevertheless, most of the commercial applications of bromelain do not require a high purity of the enzyme except for the medical, pharmaceutical and research areas. The enzyme is normally produced using less complex processes in high tonnage for the use in a bulk production of food, feed and fabrics (Illanes, 2008). Hence, the bromelain purification process can be intuitively designed depending on the destination of the enzyme (Costa *et al*., 2014). The purification process can be employed to purify the protein of interest until to the extent required for its final purpose (Jervis and Pierpoint, 1989). An increment of 2 to 4-fold purity has been recommended by Nor *et al*. (2015) for bromelain applications in the food industry which is achievable by using membrane filtration processes.

Economic analyses

Economic consideration in the application of membrane-based processing for bromelain purification is crucial for the feasibility of the production. Based on our literature search, there is only one study by Lopes *et al*. (2012) who reported on the economic evaluation of bromelain production by membrane process. They analysed the related bromelain production cost per hour and per day in Sao Paulo, Brazil, with stipulation of concentrated enzyme value of 125 mL per hour and 1 L per day. The cost calculation includes the cost of reagent, raw material, services, water and sewage and energy involved with the indirect costs, general materials, insurance, and depreciation are not taken into consideration. An estimated cost of R$25.16 to 31.79 (US$12.68 to 16.03 - based on the conversion of R$1 = US$0.504 on May 2012) per hour and R$196.94 to 254.39 (US$99.27 to 128.23) per day has been reported to be 9 to 13 times lower than the same enzyme sold by Sigma company.

In addition, they also reported that the cost estimated in their study was 6.5 to 8.5 times smaller than the previous economic analysis report on bromelain production using liquid-liquid extraction technology. However, it is necessary to emphasize that the sale price of bromelain is not solely depend on the production cost and might be influenced by other factors such as the indirect overhead costs, demands, marketability of the product etc. which may lead to a higher market price.

Thus, a valid and current economic assessment is needed to properly guide further work towards purifying bromelain from pineapple waste for sustainable production of commercial proteases. The economical evaluation on the former matter can be further expanded on a larger scale (factory set up with a higher throughput), particularly by incorporating the investment and the capital cost as well as the payback period for cost-benefit study. The evaluation may include some of the suggested improvements on the membrane process in this review including enzyme pre-treatment, two-stage UF strategy and the usage of ceramic membranes.

Conclusion

In this review, the membrane technology was presented as a potential and attractive purification method for commercial bromelain production. The feasibility of the bromelain production on the processing and purity of the enzyme via membrane-based process has been discussed in this review based on the studied literatures. However, a few aspects such as the efficiency of the process and purity of the enzyme need to be considered before this technology can be upgraded to the commercial

Table 2. Recommended improvements for membrane processing of bromelain separation

Considerations	Recommended improvements
Various components in the feed	• To perform enzyme pre-treatment as to hydrolyze pectin and other macromolecules that might interfere during the membrane process
Efficiency of the process and purity of bromelain	• To follow the HPTFF strategy particularly using the two-stage UF system, which consisted of membranes with MWCO of two times bigger and smaller than the molecular weight of bromelain • To use ceramic membranes • To adopt other membrane processing approaches such as diafiltration, gas sparging etc. • To optimize of the related processing conditions such as TMP and cross-flow velocity. • To adjust the feed to pH 6 to 7 and operate the process at a room temperature
Economy evaluation	• To evaluate the capital and investment cost. Operation cost should be further appraise based on the recommendations given above

level. Several recommendations for improvement have been discussed through this review by adopting the successful studies on protein separation and were summarized in Table 2. Enzymatic pre-treatment can be performed as to hydrolyze pectin and other macromolecules that might interfere during the membrane process. The efficiency of the process and purity of bromelain can be improved by following the HPTFF strategy (particularly the two-stage UF system), using the ceramic membrane filters and employing the other membrane processing approaches such as diafiltration, gas sparging and static mixer. Optimizing the related processing conditions including TMP and cross-flow velocity is necessary while performing the process at pH 6 to 7 in room temperature. A valid and current economy appraisal should be executed with the inclusion of the capital and investment cost to further evaluate the feasibility of the bromelain production. Based on these recommendations, further studies are needed to improve the membrane filtration process, and will hopefully leads to the future direction of this industry.

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