

REVIEW ARTICLE

Recent Advancements of Fungal Xylanase Upstream Production and Downstream Processing

Jonathan¹, Veren Tania¹, Jessica C. Tanjaya¹, Katherine^{1*}

¹*Biotechnology Department, Indonesia International Institute for Life Sciences, Jakarta, Indonesia*

**Corresponding author. Email: katherine.k@i3l.ac.id*

ABSTRACT

Xylanase is a hydrolytic enzyme produced by fungi and bacteria utilized in various industrial applications such as food, biobleaching, animal feed, and pharmaceuticals. Due to its wide variety of applications, xylanase's large-scale industrial production has gained researchers' interest. Many factors and methods affect fungal xylanase's production in both upstream and downstream processing stages. The upstream production methods used are submerged fermentation (SmF) and solid-state fermentation (SSF), where SmF involves the usage of liquid substrates, while the SSF applies solid substrates to inoculate the microbes. The downstream processing of fungal xylanase includes extraction, purification, and formulation. The extraction methods used to extract fungal xylanase are filtration and solvent extraction. Meanwhile, the purification methods include ultrafiltration, precipitation, chromatography, Aqueous Two-Phase System (ATPS), and Aqueous Two-Phase Affinity Partitioning (ATPAP). The formulation of xylanase product is obtained in either liquid from the extraction-purification results, which can be converted to powder form using technologies such as spray drying to increase storage life. Moreover, immobilization of xylanase with nanoparticles of SiO₂ could produce reusable xylanase enzymes. Several future studies have also been suggested. This review aims to explain the upstream and downstream processes of fungal xylanase production as well as the factors that affect those processes.

Keywords: Fungal xylanase; upstream ; downstream ; formulation ; bioprocess

INTRODUCTION

Xylanase is a hydrolytic enzyme that is mainly produced by fungi, yeast, and bacteria (Khanahmadi *et al.*, 2018). It has the ability to degrade xylan, into a simple monosaccharide. This enzyme has been known to create useful products that grant positive impacts on industrial and biotechnological applications. One of the most commonly used microbes to produce xylanase is fungi. There are many

strains of fungi that can induce xylanase production, such as the *Aspergillus sp.*, *Penicillium sp.*, and *Trichoderma sp* (Steudler *et al.*, 2019). The fungal xylanase is commonly used for industrial purposes in the form of powder or liquid.

The application of xylanases can be applied in many fields including food, biobleaching, animal feed, and pharmaceutical (Bhardwaj, Kumar, & Verma, 2019; Raveendran *et al.*,

2018). Xylanase is included in animal feed as a supplement because it could assist an animal's digestion by breaking down non-starch polysaccharides. Thus, improving its nutrients digestibility (L. Zhang *et al.*, 2014). In the food field, bakery, xylanase addition can enlarge and improve the internal structure of the dough properties (Ahmad *et al.*, 2014). In paper making, the forest pulp to produce paper contains xylan. Xylanase can be used to remove the xylan by enlarging the pulp matrix's porosity and allowing trapped lignin to be released. This would create an effective penetration of chemical bleaching and increase the brightness of the paper (Gangwar *et al.*, 2014). Lastly, enzymatic hydrolysis of xylan by xylanase produces xylo-oligosaccharides (XOS), which are utilized as a prebiotic supplement to promote human gut health. Not limited to that, it also had antimicrobial and anticancer activity in it. (Aachary & Prapulla, 2011; Ahmad *et al.*, 2014; Bhardwaj, Kumar, & Verma, 2019; Motta *et al.*, 2013).

The applications of xylanase have gained the interest of researchers in the labs and the industrial field. Many researchers have tried to produce xylanase in a large-scale production due to its variety of applications, which are commonly done through submerged fermentation (SmF) and solid-state fermentation (SSF). The SmF involves the usage of liquid substrates, while the SSF involves solid substrates to inoculate the microbes (Ravichandran & R, 2012). Throughout the fermentation process, many different factors need to be optimized in order to improve the production of xylanase. Some of these factors include pH, temperature, substrate type, substrate concentration, agitation and aeration rate.

This review paper will focus on reviewing xylanase's bioprocess production using different

bioreactors and the optimal parameters used to optimize its yield. Upstream processing includes the selection of *Aspergillus sp.*, *Penicillium sp.*, *Trichoderma sp.* strain in the various substrates; fermentation strategies; and controller of the parameter (pH, temperature, aeration, agitation rate). While in the downstream processing, various extraction, purification, and formulation methods will be covered. In addition, some future studies will be recommended. ScienceDirect and Google Scholar databases were used for the purpose of the review. The keywords used are the production of fungal xylanase and fungal xylanase fermentation. Then, research publications within 10 year, excluding publications that used genetic engineering were selected for the review.

The overview of the process is depicted in Figure 1.

UPSTREAM PROCESSING

Submerged Fermentation (SmF)

Submerged fermentation is a fermentation process that occurs in which microorganisms and substrates are submerged in a liquid medium. Types of microorganisms that can utilize SmF for industrial-scale include yeast, bacteria, and fungi (Manan & Webb, 2018). SmF is often performed using a stirred tank bioreactor (STR) that offers a simple control system for parameters (pH, temperature, DO, agitation; aeration rate), and is easy to handle. However, a high volume of culture medium and substrate needed, as well as high energy consumption makes this mode more expensive (Bose & Gangopadhyay, 2013). Before performing the fermentation process, all the microorganisms used are cultured in an optimized condition and inoculated to the growth medium.

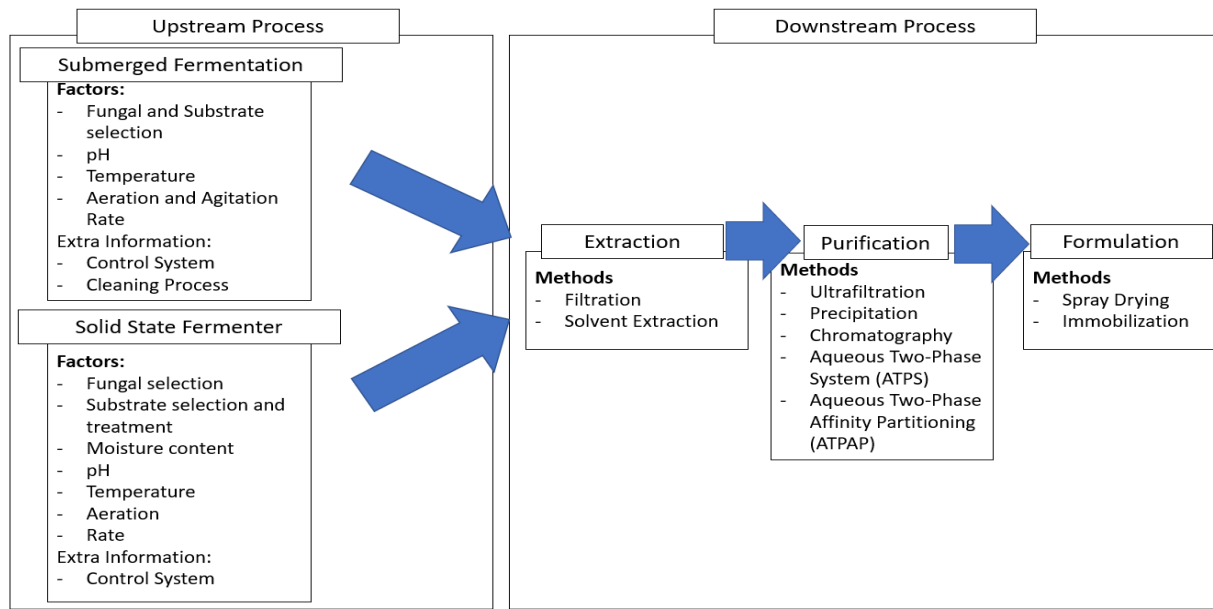


Figure 1. Overview of the fungal xylanase production

Fungal and substrate selection – Most studies reported the use of various fungi species, the majority belong to the *Aspergillus* genus, including *Aspergillus nidulans* (Abdella *et al.*, 2020), *A. niger* (Bakri *et al.*, 2011; Costa *et al.*, 2018), and *A. amowari* (Teixeira *et al.*, 2010). *Aspergillus* species is popular as it is a cellulolytic micro fungi that secrete a high amount of enzyme (xylanase) in the culture medium (Guimaraes *et al.*, 2013). Additionally, other fungi species, *Penicillium citrinum* (Ghoshal *et al.*, 2014) are compared in this review.

Pure sugar, such as glucose and maltose were preferred carbon sources due to their high enzymatic productivity (Abdella *et al.*, 2020). Due to the high cost of pure sugar, alternative fermentation substrates from agro-industrial byproducts such as wheat bran (Costa *et al.*, 2018 ; Teixeira *et al.*, 2010 , corn cob (Bakri *et al.*, 2011), and sugarcane bagasse (Ghoshal *et al.*, 2014) have been explored.

Additional steps of pretreatment for alternative substrate (wheat bran, corn cob,

and sugarcane bagasse) are essential before conducting the fermentation. Pretreatment is necessary as it enhances the availability of hemicellulose and decreases the lignin content. Therefore, xylan availability also increases. Substrates used for the fermentation are general carbon sources (glucose; maltose), wheat bran, and corn cob. The use of alternative substrates (wheat bran, corn cob, and sugarcane bagasse) is due to the high cost of pure xylan (Ghoshal *et al.*, 2014).

A study found that the *Aspergillus nidulans* yields 1572 U/mL xylanase in a synthetic medium (Abdella *et al.*, 2020). While another study found the yield of xylanase in the range of 6.22-299 U/mL by *A. niger*, *A. amowari*, and *P. Citrinium* (Bakri *et al.*, 2011; Costa *et al.*, 2018; Ghoshal *et al.*, 2014; Teixeira *et al.*, 2010). Xylanase activities measured after 96 hours fermentation using different species and substrates from various studies are summarized in Table 1.

Table 1. Comparison of Xylanase Production Using Submerged Fermentation on Various Type and Amount of Substrate by various Fungal Species.

Substrate	Microbes	Type, Time (96 h)	Enzyme Activity (U/mL)	Enzymatic Productivity / day (U/mL.d ⁻¹)	Source
120 g/L maltose + 5g/L glucose	<i>Aspergillus nidulans</i>	Batch	1250	313	(Abdella <i>et al.</i> , 2020)
180g/L maltose + 10 g/L glucose	<i>Aspergillus nidulans</i>	Fed-Batch	1193	298	(Abdella <i>et al.</i> , 2020)
180g/L maltose + 15g/L glucose	<i>Aspergillus nidulans</i>	Repeated Batch	1572	373	(Abdella <i>et al.</i> , 2020)
30g/L glucose + 1% (w/v) wheat bran	<i>Aspergillus niger</i> A12	Batch	6.22 - 8.96	-	(Costa <i>et al.</i> , 2018)
3g/L wheat bran	<i>Aspergillus awamori</i>	Batch	19.37	-	(Teixeira <i>et al.</i> , 2010)
30g/L of corn cob	<i>Aspergillus niger</i>	Continuous	162	45.5	(Bakri <i>et al.</i> , 2011)
Sugarcane bagasse	<i>Penicillium citrinum</i>	Batch	121.23-299.51	-	(Ghoshal <i>et al.</i> , 2014)

pH Control – pH is one of the factors that affect xylanase production. A low to medium pH (4 - 6.5) was found to be the most favorable in xylanase production by *Aspergillus sp* (Chen *et al.*, 2016). However, a low pH may cause enzyme activity loss if maintained for a long period (Chen *et al.*, 2016). In submerged fermentation (SmF), the pH value can be maintained using a pH sensor and acid/base addition (Imtiaz *et al.*, 2013).

Costa *et al.* (2018) reported xylanase activity of *A. niger* measured at different pH of 3 - 8. For pH comparison, this strain was treated with buffers A and B, which are 0.2 mol/L sodium citrate and 0.2 mol/L sodium phosphate, respectively. At pH 4, the xylanase enzyme activity showed the highest yield of 8.96 U/mL when treated with buffer A. However, the enzyme activity reached a stable yield of 8.72

and 8.58 IU/mL at pH 6 when the strain was treated with both buffers (Costa *et al.*, 2018). The xylanase yield in this study indicates that both buffers can be utilized to yield a high xylanase activity at an optimal pH. Teixeira *et al.* (2010) conducted a study of *A. awamori* with one control pH of 5.0-5.5 that yielded 29.02 IU/mL. Ghoshal *et al.* (2014) reported xylanase production with *Penicillium citrinum* had an optimum pH of 5 with a yield up to 299.51 U/mL.

Temperature – Temperature is a crucial parameter as it determines the microorganism's ability to ferment substrate. A high temperature was found to decrease the fungal strains' activity, damage, and may even kill the fungal strains (Benassi *et al.*, 2014). Some fungal strains may be heat-tolerant. However,

although the fungal activity won't be disturbed, the xylanase produced will have lesser enzyme activity as a high temperature may inhibit the xylanase enzyme activity (Chen *et al.*, 2016).

The optimum temperature is in the range of 30 - 60°C. A study conducted by Abdella *et al.* (2020), showed the highest xylanase activity at 37°C that yielded 1250-1572 U/mL in the repeated batch fermentation using *Aspergillus nidulans*. They also reported a similar optimum temperature of 37°C for fermentation using *Aspergillus nidulans* with batch and fed-batch modes. Optimum temperatures of 37°C were also reported for xylanase production using *Penicillium citrinum* (Ghoshal *et al.*, 2014) and *A. niger* (Bakri *et al.*, 2011; Costa *et al.*, 2018). However, a difference in optimum temperature is found in *A. amowari* which produces a lower yield of 19.37 U/mL at optimal temperature of 50 - 60°C (Teixeira *et al.*, 2010).

Aeration and Agitation Rate on K_{La} – K_{La} or volumetric mass transfer coefficient (h^{-1}) measurement is used to determine the necessary amount of the bioreactor's oxygen supply needed to support cell proliferation, which will result in product yield improvement. K_{La} values depend on the condition and are affected by the aeration, agitation rate, and impeller design (Stanbury *et al.*, 2016). *Aspergillus sp.* is a highly aerobic fermentation microbe, making the K_{La} values a vital parameter to be evaluated (Abdella *et al.*, 2020). The agitation rate should not be higher than 400 rpm as it may cause shear stress to the fungal strains, which lowers the xylanase yield. On the other hand, a low agitation and aeration rate may decrease the xylanase production too. Hence, it is crucial to keep the agitation and aeration rate at the optimum range (Bakri *et al.*, 2011).

Abdella *et al.* (2020) included the K_{La} measurement for optimizing the upstream processing parameters. They reported that the optimum aeration and agitation rate were at 400 rpm (rate per minute) and 2 vvm (vessel volume per minute) with K_{La} value of $38.55 h^{-1}$, the xylanase activities increased 166% compared to 200 rpm, 2 vvm condition. Meanwhile, they also reported that agitation at 600 rpm caused a reduction of 225 U/mL xylanase activity compared to the 400 rpm.

The reduction of activity can be due to the microbes' shear stress resulting from the high agitation rate (Abdella *et al.*, 2020). Every microorganism had its optimal condition. For instance, in *Penicillium citrinum*, aeration of 1 vvm and agitation rate of 400 rpm found to be the most optimal for xylanase production (Ghoshal *et al.*, 2014).

Control system – In submerged fermentation, the parameters that need to be controlled are pH, substrate concentration, temperature, agitation rate, and aeration rate. Each microorganism had its optimum condition to maximize its growth. Thus, control of the environment is essential to support the growth and yield obtained. It is worth noting that the control system type does not directly affect the actual production as control systems only help in adjusting the affecting fermentation parameters. Although, as different fermenters may have different control systems, preferences may exist among different users depending on the design needs.

One possible control system was reported by Abdella *et al.* (2020) with the use of a 3L Eppendorf BioFlo 115 fermenter which automatically and/or manually controls the agitation rate, aeration rate, pH, and temperature through a controller software. An acidic or basic solution needs to be provided for

pH control. The type of controller system used on the larger scale will be the combination of feedforward and feedback controller as the single use of a feedback controller may be insensitive, leading to delay of control. Therefore, the feedforward controller is used to help control the disturbance before reaching the system.

Meanwhile, the feedback control system will act as the double protection or as a backup if something happened with the feedforward controller. These controller systems are done by a PID controller. The PID controller is done by setting the setpoint to the desired target, which will give a signal to the system's output (feedback) and input (feedforward) (Hägglund, 2012).

Solid-State Fermentation (SSF)

Solid-State Fermentation is an alternative fermentation method to produce xylanase using fungi as the microorganism. SSF is suitable for producing high volume, low-value enzymes, for instance, cellulose, xylanase, and amylase. Some of the key differences between solid state fermentation and submerged fermentation is available on Table 2.

SF is considered cost-effective as it cuts down several costs in production, including the substitution of the bioreactor to tray fermenter, labor, and operational cost (Singhania *et al.*, 2015). The lower cost of the fermenter in SSF is likely due to the simpler design of tray bioreactor compared to the high-tech design needed for SmF (Table 2.). This also relates to

Table 2. Comparison of submerged fermentation and solid state fermentation
(Adapted from Manan & Webb, 2017)

Parameter	Solid State Fermentation (SSF)	Submerged Fermentation (SmF)
Free water	None	Main component of medium is water
Inoculation size	Large, more than 10%	Small, less than 10%
Production rate	High	Low
Product yield	High	Low
Mixing	Hard	Easy
Temperature control	Hard, which includes removal of metabolic heat	Easy
Product state at the end of fermentation	The medium is a wet substrate with high product concentration	The medium is liquid with low product concentration
Product extraction	Simple extraction process and less waste water generated	Complex extraction process and large amount of waste water
Water activity	Low	High
Fermentation bioreactor characteristics	Simple	High-tech design
Raw material cost	Low	High

the lower labor cost as the simple fermenter design in SSF means that handling it requires less expertise, hence lower labor cost. One of the factors that causes SSF to have less operational cost is likely the usage of agro-industrial waste as the substrate (Manan & Webb, 2017) compared to the more expensive well-defined media that is often used in SmF.

Moreover, as SSF produces less waste water (Table 2.), it can also cut down the waste treatment cost, which is another aspect of operational cost. Moreover, the application of SSF in filamentous fungi (e.g., *Aspergillus*, *Penicillium*) results in higher titer than the submerged fermentation (Singhania *et al.*, 2015). While there are also applications for SmF, SSF in this case is more suitable for the production of fungal xylanase. Aside from the previously mentioned reason, SSF is more suitable for fungal xylanase because it has been reported that SSF is more suitable for filamentous fungi, which is the main producer for fungal xylanase discussed in this review. This is likely due to the condition of the SSF that closely resembles the natural environment for filamentous fungi, such as the absence of free water in the fermenter (Manan & Webb, 2017).

Fungi selection – Various fungi species can be used to produce xylanase using the solid-state fermentation method. Many of the xylanase fungi belong to the *Aspergillus* genus, such as *Aspergillus tubingensis* (Adhyaru *et al.*, 2015), *Aspergillus niger* (Costa *et al.*, 2018), and *Aspergillus oryzae* (Pirota *et al.*, 2013). However, aside from the *Aspergillus* genus, fungi from other genus may also be utilized, such as from the *Penicillium* genus and *Trichoderma* genus. One interesting finding suggests that co-culture using two different microorganisms may give a higher xylanase yield than a monoculture production. Dhillon *et*

al. (2011) discovered that 2710.6 IU/gds xylanase was produced using a mixed culture of *Trichoderma reesei* and *Aspergillus niger*, which is higher than using *Aspergillus niger* (2604 IU/gds) and *Trichoderma reesei* (2467.5 IU/gds) as a single culture. The various fungi used to produce xylanase using solid-state fermentation can be seen in Table 3.

Substrate selection and treatment – Much agro-industrial waste can be used to produce xylanase using solid-state fermentation. Some examples include sorghum straw, wheat bran, corn cobs, sugarcane bagasse, and many more (Table 3). It may be difficult to compare which substrate is the best to be used to produce xylanase, as the compiled research utilizes different substrates and microorganisms. However, there are some studies that directly compared different substrates on one microorganism. One such example is a study by Pandya & Gupte (2012) who compared different substrates such as wheat bran, wheat straw, sugarcane bagasse, and rice bran. They found that *Aspergillus tubingensis* produces the highest yield when using wheat straw as substrate, yielding as high as $1,478 \pm 11$ U/g xylanase compared to other substrates sugarcane bagasse, which only yields 17 ± 3 U/g xylanase. Different results could be found in different research, such as a study conducted by Ghoshal *et al.* (2016). They found that *Penicillium citrinum* can produce xylanase as high as 2,298 U/g using sugarcane bagasse. Therefore, it is important to conduct a preliminary study to determine which substrate should be used.

However, aside from selecting the substrate itself, there are other optimization methods that could be done to improve the results further. One such method is the pretreatment of the substrates. Bandikari *et al.* (2014) found

Table 3. Comparison of Xylanase Production Using Solid State Fermentation on Various Agro Industrial Waste by various Fungal Species.

Substrate	Microbes	Reactor	Maximum Yield	Source
Sorghum Straw	<i>Aspergillus tubingensis</i>	Flask	4,105 (U/g)	(Adhyaru <i>et al.</i> , 2015)
Dry wheat bran	<i>Aspergillus niger</i>	Flask	9.65 (U/mL)	(Costa <i>et al.</i> , 2018)
Pretreated Corn Cob + Pineapple Peel	<i>Trichoderma koeningi</i>	Flask	2,869.8 ± 0.4 IU/g	(Bandikari <i>et al.</i> , 2014)
Tomato Pomace	<i>Aspergillus awamori</i>	Plate-Type	195.92 ± 11.0 IU/gds	(Umsza-Guez <i>et al.</i> , 2011)
Wheat Bran	<i>Aspergillus oryzae</i> P6B2	SSF lab-Scale System	2,830.7 IU/g	(Pirota <i>et al.</i> , 2013)
Wheat bran	<i>Aspergillus niger</i> CCUG33991	Tray Solid State Fermentation	2,919±174 U/g	(Khanahmadi <i>et al.</i> , 2018)
Sugarcane Bagasse	<i>Penicillium citrinum</i> MTCC 9620	Flask	2,298 U/gds	(Ghoshal <i>et al.</i> , 2016)
wheat straw	<i>Aspergillus tubingensis</i> JP-1	Flask	6,887 ± 16 U/g	(Pandya & Gupte, 2012)
Oil Palm Empty Fruit Bunches (OPEFB)	<i>Aspergillus niger</i> USM SD2	Flask	3,246 ± 18 U/g	(Ajijolakewu <i>et al.</i> , 2017)
Rice Straw and Wheat Bran	<i>Trichoderma reesei</i> Rut C-30 & <i>Aspergillus niger</i> BC-1	Tray Solid State Fermentation	3106.34 IU/gds	(Dhillon <i>et al.</i> , 2011)
<i>Jatropha curcas</i> seed cake	<i>Aspergillus niger</i> FGSCA733	Flask	6,087 U/gds	(Ncube <i>et al.</i> , 2012)
Coffee Pulp	<i>Penicillium</i> sp. CFR 303	Flask	23,494 U/g	(Murthy & Naidu, 2012)
Babassu cake	<i>Aspergillus awamori</i> IOC-3914	Fixed-Bed Bioreactor (Pilot-scale)	31.1 ± 1.3 U/mg	(Castro <i>et al.</i> , 2015)

that alkaline pretreatment using 2% NaOH can increase xylanase production yield. The example within the study showed that unoptimized xylanase production using pretreated corn cob could yield as high 1267.3 ± 0.6 IU/g xylanase compared to the untreated

corn cob, which only yielded 810.7 ± 0.1 IU/g xylanase. Another form of possible pretreatment is heat pretreatment. Murthy & Naidu (2012) discovered that steaming coffee pulp at 121°C for 30 minutes can increase xylanase production of *Penicillium sp* to 23,494

U/g when compared to 14,765 U/g yield of untreated coffee pulp. The positive effect of substrate pretreatment on xylanase yield has been connected to various beneficial effects such as an increase of surface area in substrate and partial depolymerization of complex carbohydrates, among other effects that increase the substrate availability (Bandikari *et al.*, 2014; Murthy & Naidu, 2012).

Another important factor is the substrate size. Several studies have discovered that there are optimal substrate sizes for xylanase production, although this may differ depending on the substrate and the microorganism used. Khanahmadi *et al.* (2018) found that the optimal size for wheat bran to produce xylanase using *Aspergillus niger* was between 0.6mm and 0.3mm. Murthy & Naidu (2012) discovered that the optimal coffee pulp size for xylanase production was 1.5mm, which yielded 14,841 U/g compared to other sizes such as 6,020 U/g yield produced by 0.5mm coffee pulp. Substrate that is too small may cause poor growth as it limits aeration, while substrate that is too big may limit area for fungal growth and poor heat conductivity (Khanahmadi *et al.*, 2018; Murthy & Naidu, 2012). An optimal substrate size enhances yield by providing better aeration.

Substrate supplementation can also enhance xylanase production. Bandikari *et al.* (2014) researched various combinations of agro-waste (such as wheat straw, rice straw, corn cob, and others) and fruit peels such as pineapple peel among others to see which combination may give the highest yield. The highest yield was achieved by a combination of pretreated corn cob and pineapple peel, which produce 2,869.8 ± 0.4 IU/g xylanase when compared to only pretreated Corn Cob, which produces 1267.3 ± 0.6 IU/g xylanase. A similar positive effect of substrate supplementation was also found by Ncube *et al.* (2012) in which *Jatropha curcas*

seed cake supplemented with 10% *Hyperhaenia sp* produce five times xylanase compared to *Jatropha curcas* seed cake as a sole substrate. Similar findings were also discovered by Dhillon *et al.* (2011), where 2859.6 IU/gds (IU/gram of solid substrate) was produced using a 3:2 ratio of rice straw and wheat bran, which is higher than 2710.6 IU/gds xylanase when rice straw is used as the sole substrate.

Moisture Content – Moisture content is also an important factor that can enhance xylanase yield. Initial moisture content is important because every microorganism needs water. Low moisture content inhibits growth due to lack of water, whereas moisture content that is too high can reduce production by reducing oxygen availability (Khanahmadi *et al.*, 2018). The optimal initial content may vary depending on the microorganism used, but generally, it would be in the range of 50%-80%. Pirotta *et al.* (2013) determined 80% moisture content was optimal for xylanase production using *Aspergillus oryzae*, whereas Khanahmadi *et al.* (2018) determined that 70% moisture content was optimal for *Aspergillus niger*. Murthy & Naidu (2010) found that 50% moisture content was optimal for *Penicillium sp*, giving a yield of 13,230 U/g xylanase. They also found that when using 60-80% moisture content, the xylanase yield is lower than 50% moisture content, although it is still significantly higher than using 40% initial moisture content, which only yields less than 7,000 U/g xylanase.

Temperature – Temperature also plays an important role in xylanase production. The optimal temperature for fungal xylanase may differ depending on the microorganism used, but most of the research reviewed in this paper suggests that the optimal temperature range is

around 28-30°C. Some fungi may tolerate fluctuation in temperature, such as in the research by (Pirota *et al.*, 2013) in which the yield remains above 1400 U/g in the temperature range of 28-32°C but falls to around 800 U/g starting from 35°C. Murthy & Naidu (2012) an optimal temperature of 30°C for *Penicillium sp.*, with a yield of 15,841 U/g, while increasing the temperature to 32°C leads to a decrease of yield to 11,026 U/g and reducing the temperature to 27°C further decrease the yield to 5,641 U/g. Adhyaru *et al.* (2015) reported an optimum temperature of 40°C for xylanase production using *Aspergillus tubingensis* with a yield of 1,998 ± 12 U/g, although overall it maintains a yield of above 1,000 U/g in the temperature range of 25-55°C.

Initial pH – Much like temperature, pH can also affect fungal xylanase production. Unlike temperature, however, there is no way to adjust the pH once the fermentation process begins in solid-state fermentation. This holds for both static fermentation in a flask or fermentation in a solid-state fermenter. pH can be maintained by adding a buffer as a moisturizing agent for the substrate. Interestingly, agro-industrial waste also has some buffering capability (Murthy & Naidu, 2012).

The optimum initial pH may differ depending on many factors, including the microorganism used. Some research discovers a pH range of 5-6 to be the optimum pH (Adhyaru *et al.*, 2015; Ghoshal *et al.*, 2016; Murthy & Naidu, 2012; Pandya & Gupte, 2012). Ncube *et al.* (2012) reported an optimum pH of 3 for xylanase production using *Aspergillus niger*.

Aeration – Aeration has been reported to have a positive impact on xylanase production. One of the best representations is reported by

Umsza-Guez *et al.* (2011), who reported a xylanase yield of 195.92 ± 11.0 IU/gds when using water-saturated forced aeration compared to 104.43 ± 5.2 IU/gds (IU/ gram of solid substrate) yield obtained from conical flask (static aeration) condition. Pirota *et al.* (2013) also reported a higher yield of xylanase using instrumented lab-scale fermenter (forced aeration) compared to Erlenmeyer flask (static aeration) for all experiments conducted.

Pilot-Scale Fermentation – Most of the papers reviewed regarding solid-state fermentation conducted the experiment in the lab-scale fermenter, whether in a flask or tray fermenter. However, there is also a publication that reported the usage of a pilot-scale solid-state fermentation using a fixed-bed fermenter (Castro *et al.*, 2015). The reactor had a working capacity of 1.8L and produced up to 31.1 ± 1.3 U/mg Xylanase. The fixed-bed reactor was suggested to be a cost alternative to tray bioreactor. However, it has some underlying problems such as non-uniform fungal growth that needs to be solved by further optimization.

Control System – There are many types of bioreactors that can perform solid-state fermentation, and the control system for each of these bioreactors may differ from one another. Generally, the controllable aspects of this type of fermentation are temperature, aeration, and humidity.

Pirota *et al.* (2013) utilized a lab-scale solid-state lab-scale bioreactor composed of 16 columns placed in a water bath as described by Farinas *et al.* (2011). The bioreactor used on-line measurement to control airflow rate and inlet air relative humidity using a feedback control system. It is also possible to regulate the temperature using the water bath as the bioreactor also included a temperature sensor.

Khanahmadi *et al.* (2018) utilized a tray bioreactor chamber filled with trays to perform the fermentation. The bioreactor supplied saturated air inflow controlled by a rotameter. The temperature was monitored using temperature probes and adjusted with parallel surface aeration, which involved adjusting the temperature of the saturated airflow.

The fixed-bed bioreactor used by Castro *et al.* (2015) possesses four thermal sensor ports. The bioreactor also has a jacketed air humidifying chamber maintained at a specific temperature to supply humidified air to the reactor.

DOWNSTREAM PROCESSING

Downstream processing is described as various stages that occur after the fermentation stage, which in this case covers extraction, purification, and packaging. The downstream process is highly crucial for biopharmaceutical (antibiotics, vitamin, vaccine) and industrial enzymes, as the products from upstream are processed to meet the purity and quality requirements. This process is also costly, therefore, cost-efficient methods need to be developed (Labrou, 2014). Various techniques such as organic solvent extraction, ion exchanger, chromatography, and gel filtration have been used commercially (Fakhari *et al.*, 2017). Several new methods had been proposed as a cost-effective alternative compared to previous methods.

Extraction

Protein extraction is the process of releasing the targeted protein, which in this case is xylanase, from the biological matrix (Łojewska *et al.*, 2016). The extracted fungal xylanase will

be in the form of a crude enzyme, where it can be used for testing, or it can be further purified depending on the needs. The amount of crude xylanase obtained depends greatly on the methods of extraction, the type of fungi used, and other factors such as temperature, agitation, extraction time, and buffer (Adhyaru *et al.*, 2015). Most studies reviewed in this paper mentioned the use of filtration and solvent extraction to obtain crude xylanase.

Filtration – Filtration is often used to obtain crude xylanase from Submerged Fermentation. Filtration is commonly used to remove impurities such as other proteins or any other molecules besides xylanase. Filtration in large scale production works by having a barrier with a set size of pores to act as the filter, allowing molecules with size smaller than the filter pores size to pass through while retaining the rest. One important characterisation of filtration is Molecular Weight Cut Off or MWCO (Singh, 2015). MWCO has been described as the molecular weight at which 90% of the molecules are retained or rejected by the membrane (Singh, 2015). This means that molecules that are above the MWCO will be retained or considered as the retentate. Xylanase is usually located in the filtrate, as long as the Molecular Weight Cut Off is more than 10 kDA as xylanase size is above 10 kDA (Teixeira *et al.*, 2010). It is worth noting that most of the studies reviewed in this paper do not discuss filtration methods in detail, only using them as a method for laboratory scale analysis. This makes it hard to determine the best method used in large scale production of fungal xylanase. The lack of filtration study from the last 10 year studies may suggest that filtration is an established method and no recent advancement was reported. The studies covered in this paper often used Whatman filter

paper to obtain the crude xylanase used (Bhardwaj & Verma, 2020; Garai & Kumar, 2013) while other studies may use centrifugation after the filtration to obtain the cell free supernatant that can be considered as the crude xylanase (Bhardwaj, Kumar, Agarwal, *et al.*, 2019).

One strategy that might be used in large scale production of xylanase is ultrafiltration. However, ultrafiltration is often considered as a purification rather than extraction method and therefore will be described further in the purification section in this paper.

Solvent Extraction – Solvent extraction is often used to extract used xylanase from solid support from solid state fermentation. Ideally, solvent extraction can extract the desired enzyme selectively and completely within minimum contact time (Adhyaru *et al.*, 2015). Solvent extraction is normally conducted by adding appropriate solvents to the cultivated solids, followed by agitation within a preset temperature and time. There are several factors that affect the result of solvent extraction. The factors include: choice of solvents, solvent temperature, solvent volume, extraction time, extraction agitation, and extraction temperature (Adhyaru *et al.*, 2015, 2016; Pal & Khanum, 2010).

Adhyaru *et al.* (2015) performed xylanase extraction from *A. tubingensis* with different solvents, namely citrate buffer, sodium citrate buffer, double-distilled water, phosphate buffer, Tris-HCl buffer, tween-80 and NaCl within sorghum straw substrate. Xylanase extraction with sodium citrate buffer was reported to have higher enzyme recovery (3.573 ± 22 U/g) compared to other solvents. The difference of enzyme recovery may be affected by the *A. tubingensis* properties and its suitability with different buffers. Other factors

that affect enzyme recovery would be the extraction solvent volume and time. Adhyaru *et al.* (2015) reported an increase in enzyme extraction (3.769 ± 22 U/g to 3.951 ± 17 U/g) at 12:1 extraction solvent ratio and 90 min of extractant time. By using small solvent volume, the enzyme would not require extensive energy to recover.

Similar to Adhyaru *et al.* (2015), Pal & Khanum (2010) utilized deuterium depleted water (DDW), Tris-HCl buffer, citrate buffer, tween-80 and NaCl as extraction solvents at different temperatures from moldy WB-SBC medium. This study reported higher xylanase recovery of 2885 IU/gds at 37°C from *A. niger* using DDW solvent. This may be due to the reduced substrate's interactive forces with xylanase, which is caused by higher dielectric constant of water compared to other solvents.

Recently, Adhyaru *et al.* (2016) conducted a study on *A. tubingensis* to obtain the optimal extraction solvent volume, extraction time, extraction speed, and temperature through a statistical approach. This study reported a high xylanase activity of 5177.23 U/g at 12 - 13 mL/g extractant volume, 110 - 112 min extraction time, 160 - 165 rpm extraction speed, and 39°C - 40°C temperature. The extracted xylanase activity at optimal variables was reported to have increased from its initial activity (4061.32 U/g) by 1.3-fold. The increase in xylanase activity is associated with the prevention of denaturation that resulted from the detachment of xylanase on the substrate. Furthermore, the study conducted a repeated extraction to compare xylanase recovery when extracted with optimal variables and non-optimal variables statistical models. They reported that the xylanase extracted under optimal variables reached 90% xylanase recovery.

Purification

The purification process is necessary to remove undesired molecules from the targeted protein. The type of purification method used depends on the enzyme's physical and chemical properties (Lee, 2017).

Two parameters will be used in this review to describe the results of purification. The first parameter is yield or recovery yield (%), which indicates the total activity or amount of enzyme after the purification divided by the total protein (extract) or activity before the purification. The second parameter is the purification factor or purification fold, which indicates the enzyme's specific activity after purification divided by the specific activity of the enzyme before purification.

Many purification methods have been developed, such as ultrafiltration, precipitation, chromatography, and aqueous two-phase partitioning (otherwise known as aqueous two-phase system). Each of these methods have their own advantages and disadvantages.

Ultrafiltration (UF) – Ultrafiltration is one of the starting methods of purification. The term 'ultrafiltration' itself is designated for usage of filters with pore size between 0.001–0.05 μm , and one of its applications is to concentrate protein (Singh, 2015). Ultrafiltration is often used as the first method of purification before methods that can further protein such as chromatography are used (Adigüzel & Tunçer, 2016; Dhiman *et al.*, 2012, 2013; Teixeira *et al.*, 2010). They can be used to filter supernatant directly from centrifugation of fermentation broth (Adigüzel & Tunçer, 2016; Dhiman *et al.*, 2012, 2013) or to further filter (ultrafilter) culture supernatant that has been filtered beforehand (Teixeira *et al.*, 2010).

One of the approaches of using ultrafiltration is by directly using filters with MWCO smaller than the xylanase, so the xylanase can be found in the retentate, which is also concentrated. This approach is performed by Adigüzel & Tunçer (2016), Dhiman *et al.* (2012), and Dhiman *et al.* (2013), who utilized UF with MWCO of 10 kDa. Adigüzel & Tunçer (2016) managed to achieve a purification factor of 1.2 compared to enzymes in the culture supernatant. Dhiman *et al.* (2013) and Dhiman *et al.* (2012), on the other hand, only reported that UF was used to concentrate the enzyme before proceeding to the next purification method such as chromatography, with the result from the UF used as the standard, making it hard to determine the purification fold of the UF compared to the enzyme in the culture supernatant. The 10 kDa cut-off managed to retain the xylanase because the xylanase MW was reported to be above 10 kDa.

The other approach of using UF is to use a filter size that allows the xylanase to permeate the filter (also called ultrafiltrate) (Teixeira *et al.*, 2010). Teixeira *et al.* (2010) utilized two-step UF where the first filter had the size of 300 kDa (permeate yield = 87.14 %, permeate purification fold = 1.21), and the permeate of the first filter was further filtered with a 100 kDa filter resulting in permeate (yield = 69.48% and PF = 1.43) and retentate (yield = 8.37% and PF = 0.70) with high activity though the permeate had higher activity as xylanase size was reported to be around 46-16 kDa. The result from the ultrafiltration can be purified further using chromatography.

Precipitation – Protein precipitation is said to be one of the most important steps in early downstream processing systems. This method's main objective is to precipitate the desired enzyme from unwanted components, including

small ions (Ho & Ilyia, 2015). There are several methods to perform precipitation, but in this review, 3 will be covered, which include: using ammonium sulfate (Bhardwaj, Kumar, Agarwal, *et al.*, 2019; Ding *et al.*, 2018; Ho & Ilyia, 2015), ethanol (Costa *et al.*, 2018), and polyelectrolyte or polymer-protein complex formation (Podestá *et al.*, 2019).

Precipitation by ammonium sulfate can be considered as an established method for enzyme purification. The main mechanism behind it is the 'salting out' phenomenon in which in an increased salt concentration, protein solubility decreases. The addition of salts to the solution increases the surface tension of water, while also forcing the proteins to group together (eventually leading to precipitation) to free up water that may be bound to protein to make the condition thermodynamically favorable (Wingfield, 2001). Ammonium sulfate is often used due to its higher solubility compared to other phosphate salts, which makes its ability to increase water surface tension and protein stability the highest (Wingfield, 2001). This makes ammonium sulfate an ideal reagent for salting out (Wingfield, 2001).

One of the most important factors that affect precipitation by ammonium sulfate is the concentration of the salt/ammonium sulfate itself. Ho & Ilyia (2015) used 70% ammonium sulfate to purify xylanase from *A. brasiliensis*, resulting in a purification fold of 1.05 and recovery yield of 86.02% compared to its crude enzyme. Bhardwaj, Kumar, Agarwal, *et al.* (2019) and Ding *et al.* (2018), on the other hand, used found that 60% ammonium sulfate was optimal for xylanase purification of xylanase *Aspergillus oryzae* LC1 (purification fold 4.1 and recovery yield of 21%) and *Pichia stipitis* (purification fold 11.7 and recovery yield of 88.2%). However, it is worth noting that

different species and strains used for production affect its enzyme production and activity, which also affect its specific activity in the purification stage (Ho & Ilyia, 2015). While this method is commonly used, it has some drawbacks such as the multi-stage nature of this method, which may cause loss of yield of the product, not to mention the need for high concentration of ammonium sulfate also contribute to the cost of the processing (Bhardwaj, Kumar, Agarwal, *et al.*, 2019). Hence, the development of alternative precipitation methods is recommended.

Precipitation using ethanol has been proposed as an alternative precipitation method by Costa *et al.* (2018), although the research is still on a laboratory scale. Ethanol precipitation is proposed because it is considered as a low-cost renewable material and complies with the biorefinery principle. Ethanol itself is an established important precipitant in the industry. However, the appropriate alcohol concentration is required to optimize the yield while minimizing the cost and risk of protein denaturation. The study investigated the appropriate concentration of ethanol and pH for optimal xylanase purification from *A. niger*. The study discovered that ethanol concentration of 85% and pH of 5.5 is optimal for xylanase purification from both SSF (65% activity recovered and 0.74 purification factor) and SMF (79% activity recovered and 1.03 purification fold) method. The study used an incubation time of 15 minutes, which has already recovered most of the enzyme activity. However, increasing the incubation may likely increase the activities recovered as well as the purified factor, although further research is recommended to investigate the drawbacks of a longer incubation period.

Recently, Podestá *et al.* (2019) suggested an alternative method of xylanase precipitation

produced by *A. niger* by forming insoluble protein-polymer complexes, otherwise called polyelectrolyte precipitation. Polyelectrolytes are essentially polymers with a high ionic charge that can bind with the desired protein. This method is proposed as a low cost and eco-friendly method as polyelectrolyte can come from natural sources. The polymers are also non-toxic. In this study, there are 2 polyelectrolyte studies. The first polymer is cationic chitosan (CHS), a biopolymer made from chitin's deacetylation (a component of crustacean exoskeleton). The second polymer is anionic carrageenan (Carr), a hydrophilic linear sulfated polysaccharide obtained from red seaweed cell walls. Several important factors affect this method, including: pH, incubation time, incubation temperature, and polymer concentration. The study reported that precipitation with Carr has a higher Purification Factor (PF) compared to precipitation with CHS. Precipitation with Carr resulted in PF of 9 using 0.5% w/v Carr at pH of 7, and incubation for 60 minutes at 8°C. Precipitation with CHS resulted in PF of 5.6 using 0.05% w/v CHS at pH of 8, and incubation for 60 minutes at 8°C.

Chromatography – Chromatography is another purification method, usually employed after ammonium precipitation or ultrafiltration to increase the purification factor (Adigüzel & Tunçer, 2016; Ding *et al.*, 2018; Ho & Ilyia, 2015). The most commonly used chromatography are anion exchange column chromatography and gel filtration chromatography. Anion exchange column chromatography essentially utilized positively charged resin to trap the negatively charged xylanase in the chromatography column before eluting it later on with an elution buffer (Ho & Ilyia, 2015). Gel filtration chromatography is also known as size exclusion chromatography,

which means it can separate xylanase from other components by using size difference. Both of the chromatography can be used in sequence, which is also called two-step chromatography. It is worth noting that column chromatography required prior equilibration with buffers.

Ho & Ilyia (2015) utilized Diethylaminoethanol (DEAE) sepharose anion column chromatography to further purify the xylanase precipitate (from ammonium sulfate precipitation) with NaCl as the elution buffer. The highest activity was achieved with 0.3M of NaCl with a purification fold increase from 1.05 to 1.73 while reducing the recovery yield from 86.02% to 2.77%. The process was further continued by purifying the enzyme using Sephadex G-75 gel filtration column chromatography. The enzyme was eluted with 0.05 M sodium phosphate buffer (pH 5.3). This process further increases xylanase's purification fold from 1.73 to 2.39 and further decreases the recovery yield from 2.77% to 1.36%.

Ding *et al.* (2018) utilized a similar method, but with reversed order of two-step chromatography and different elution buffer compared to Ho & Ilyia (2015). They first utilized gel filtration column Sepharose CL 6B to further purify the enzyme precipitate (from ammonium sulfate precipitation) using 0.05 M Tris-HCL buffer (pH 8) as the elution buffer. The result was then dialyzed to concentrate the enzyme. This resulted in an increase of purification factor from 11.7 to 33 while reducing its recovery yield from 88.2% to 14%. They later used ion-exchange column Q Sepharose Fast Flow with 1M NaCl as an elution buffer. This further increased the purification from 33 to 38.7 though it reduces the recovery yield to 5.7%.

The research of Ding *et al.* (2018) and Ho & Ilyia (2015) have shown how a two-step

chromatography can further improve the purification fold of the enzyme at the expense of the recovery yield. Bhardwaj, Kumar, Agarwal, *et al.* (2019) conducted a study using ion-exchange chromatography and size chromatography as single-step chromatography without prior ammonium precipitation to examine each method's efficiency. They reported that using ion-exchange chromatography (DEAE-Sephadex A-50) with NaCl as an elution buffer resulted in 3-fold purification and 31.9% recovery yield using 600mM NaCl. They also reported that using gel filtration chromatography (Sephacrose G-100) with 50 mM sodium acetate buffer (pH 5.0) resulted in a 6.6 purification fold and 78.7% recovery yield. Their result showed how only using one chromatography method resulted in a lower purification factor.

It is possible to use even more than two steps chromatography where even between the chromatography step, the xylanase is purified further using ultrafiltration and dialysis, which was performed by Dhiman *et al.* (2013) and Dhiman *et al.* (2012). However, it is worth noting that such multi-step purification to achieve high purity is more likely to be suited for analysis rather than production, as multi-step purification is costly and causes loss of yield, which is undesirable for mass production (unless a really high purity product is required).

Aqueous Two-Phase System (ATPS) – ATPS is a system that utilized mixed immiscible polymer-polymer or polymer-salt solutes in a hydrophilic solvent above critical concentration (Bhardwaj & Verma, 2020; Garai & Kumar, 2013). This method can separate the desired protein from contaminant protein in separate phases according to the biomolecular properties of the proteins themselves (Bhardwaj & Verma, 2020). This method's single-step nature makes it more

advantageous to the conventional multi-step purification (precipitation - dialysis - chromatography) (Bhardwaj & Verma, 2020; Garai & Kumar, 2013). It is simple, cost-effective, can be used in a cyclic process, as well as producing high yield and high purification factor at high velocity, not to mention it reduces the chance of protein denaturation because it often uses water as the solvent (Bhardwaj & Verma, 2020; Garai & Kumar, 2013).

There are several factors that can affect ATPS. The first factor is the selection of the hydrophilic solutes that will make up the two phases. Bhardwaj, Kumar, Agarwal, *et al.* (2019) compared two different ATPS system, one using a mixture of water and Triton X-114 (a non-ionic detergent) with the method described by Kocabaş *et al.* (2015), and the other system used polyethylene glycol (PEG) and salts to form the two phases. They reported that optimized ATPS with PEG and salts produced better results (PF = 13 & yield = 86.8%) compared to optimized ATPS with Triton X-114 (PF = 3.3 & yield = 69.4%).

The next factor that may affect ATPS, especially the system that utilizes PEG, is the PEG's molecular weight. There are various PEG molecular weights (MW) that can be utilized, such as 4000, 6000, and 8000. Optimization of PEG MW typically utilized Response Surface Methodology (RSM) using the Box-Behnken design approach along with other parameters such as Salt and PEG concentration (Bhardwaj, Kumar, Agarwal, *et al.*, 2019; Bhardwaj & Verma, 2020; Garai & Kumar, 2013). Bhardwaj, Kumar, Agarwal, *et al.* (2019) reported PEG 8000 was optimal for xylanase from *Aspergillus oryzae* LC 1. Similar choice was reported by Bhardwaj & Verma (2020) who also used PEG 8000 for xylanase from *Aspergillus oryzae* LC1. Meanwhile, Garai & Kumar (2013) reported PEG

4000 was optimal for xylanase from *Aspergillus candidus*.

The next factor that needs to be optimized is the salt choice to go along with the PEG. There are many sulfate salts that can be an option (e.g. $ZnSO_4$, $MnSO_4$, $FeSO_4$, $CuSO_4$). Optimization of salts can be done either by the RSM using Box-Behnken design approach (Bhardwaj, Kumar, Agarwal, *et al.*, 2019), or it can be screened manually (Bhardwaj & Verma, 2020; Garai & Kumar, 2013). Bhardwaj, Kumar, Agarwal, *et al.* (2019) as well as Bhardwaj & Verma (2020) much like with PEG MW selection also choose similar salt which was $MgSO_4$. Meanwhile, Garai & Kumar (2013) reported from screening that NaH_2PO_4 produced better result (PF = 2.40, yield = 81.11%) compared to $MgSO_4$ (PF = 1.46, yield = 66.05%) for xylanase purification from *Aspergillus candidus*.

PEG and salt concentration are the last factors that needed to be optimized. It is usually optimized using RSM with Box-Behnken design approach (Bhardwaj, Kumar, Agarwal, *et al.*, 2019; Bhardwaj & Verma, 2020; Garai & Kumar, 2013). Bhardwaj, Kumar, Agarwal, *et al.* (2019) as well as their future work by Bhardwaj & Verma (2020) once again reported similar findings of using 11.3% (w/w) PEG and 22.5 % (w/w) salts to produce optimal results (PF = 13, yield = 86.8). Meanwhile, Garai & Kumar, (2013) reported PEG concentration of 8.66% (w/w) and salt concentration of 22.40% (w/w) for optimal xylanase purification from *Aspergillus candidus* (PF = 3.03, yield = 88.1%).

It is likely that each parameter is optimized for different xylanase producers (fungal species) as well as their production. This is proven by the research performed by Bhardwaj, Kumar, Agarwal, *et al.* (2019) who reported the same parameter when similar research with the same fungal strain and production method was carried out in 2020 (Bhardwaj & Verma, 2020).

Meanwhile, Garai & Kumar (2013) who worked with *Aspergillus candidus* reported different parameters altogether. Hence, it is recommended to perform a preliminary study when working with other fungal strains or even when using different production (upstream) methods.

Aqueous Two-Phase Affinity Partitioning (ATPAP)

ATPAP is essentially a modification of ATPS system with the addition of a ligand that in the form of modified polymer used for the ATPS system (Fakhari *et al.*, 2017). Fakhari *et al.* (2017) propose the first usage of metal affinity partitioning with ATPS (essentially ATPAP) for fungal xylanase purification from crude enzyme extract. They utilized PEG-IDA- Cu^{2+} as the ligand, which is essentially PEG-modified with Iminodiacetic acid (IDA) and Copper Iron (Cu^{2+}). Metal affinity partitioning allowed for rapid purification of proteins of interest that contain histidine, cysteine, and tryptophan on the surface (Fakhari *et al.*, 2017). Fakhari *et al.* (2017) used PEG 6000 and Na_2SO_4 salts along with PEG-IDA- Cu^{2+} and the crude enzyme extract. This method also introduces new parameters to be optimized which are the ligand concentration (PEG-IDA- Cu^{2+}), pH, and amount of enzyme loaded. Increasing the ligand concentration was reported to positively impact the recovery yield but with a negative impact on the specific activity (or purification factor) (Fakhari *et al.*, 2017). pH was reported to affect the electrochemical interaction in the system, therefore it is important to find the optimum pH for designing the purification procedure. Fakhari *et al.* (2017) reported that increasing pH has a positive impact on both the yield and purification factor. The last factor is the amount of crude enzyme loading, in which it has been reported that it has a positive effect on xylanase purification factor (Fakhari *et al.*, 2017). It is

worth remembering that all factors need to be optimized depending on the needs, as Fakhari *et al.* (2017) reported that using RSM there were 2 optimal points where one point results in higher yield and the other results in higher purification factor.

Formulation

The last part of downstream processing is the formulation or finishing step. This process is implied to maintain the activity, stability of the product during storage and distribution (Sutton & Barr, 2018). Xylanase final products are in the form of liquid or powder. During this step, extracted-purified liquid xylanase is stored in 4°C or 30°C with an additional stabilizer to retain its activity for storage or distribution purposes (El-Sherbiny & El-Chaghaby, 2012). One of the methods to obtain the xylanase powder is by applying spray drying (Sutton & Barr, 2018). Other methods, such as enzyme immobilization, are commonly used for the production of re-used enzymes (Austin, 2018). Therefore, this method is studied for xylanase immobilization by Dhiman *et al.* (2012) and Dhiman *et al.* (2013).

Spray Drying – Spray drying is the widely used method for the formulation of animal feed, and the food industry, which converts suspension to powder form (Huang *et al.*, 2017). This technique utilized the atomization of the medium to droplets that will evaporate and moisture elimination in the heated atmosphere (Santos *et al.*, 2018). Formulation of the enzyme can be done directly from its crude enzyme or from the extracted-purified product. To achieve the powder formation, parameters such as the temperature of the inlet and outlet, type of carries, and binders should be in suitable conditions (Gupta *et al.*, 2014). Failing to

achieve the optimum temperature will result in unsuccessful powder formation and low quality, quantity, and enzyme stability. While unsuitable carries and binder combination will affect the half-life significantly due to the denaturation at high temperature on the spray drying processes (Gupta *et al.*, 2014).

Gupta *et al.* (2014) had tried several combinations of binder and carries in the crude enzyme, and the MgSO₄ and malt extract showed the greatest enzyme specificity percentage up to 99.1%. The higher the specificity indicates a more stable product formed. Data showed a reduction of 27.5 folds of xylanase half-life compared to the one without using binder and carrier combination. All of these data are performed at the optimum temperature of 140/62.5°C for the inlet and outlet, respectively. Increasing the temperature resulted in low quality and reduction of powder yield. Moreover, Gupta *et al.* (2014) estimated that spray drying helps to maintain storage stability with a half-life of 202 days. Determination of storage stability had been performed by storing the product in a McCartney bottle at 30°C for measuring residual xylanase and specific activity. Xylanase powder is packaged either in the plastic packaging or drum barrel, depending on the purposes and buying quantity.

Immobilization – The immobilization process is the entrapment of enzymes in a distinct matrix. Several methods for enzyme immobilization are adsorption, covalent bonding, entrapment, cross-linking, and encapsulation. Recently, Dhiman *et al.* (2012) and Dhiman *et al.* (2013) had demonstrated the use of covalent bonding processes on xylanase production. Covalent immobilization involves forming the covalent bonds between enzymes and carries (water-

insoluble) to create a stable complex (C. Zhang & Xing, 2011).

Two studies were conducted related to xylanase production's immobilization from *Armillaria gemina* (Dhiman *et al.*, 2012) and *Pholiota adiposa* (Dhiman *et al.*, 2013) with SiO₂ nanoparticle through covalent bonding. Factors that affect the efficiency of immobilized enzymes are temperature, pH, agitation speed, incubation time. Both studies reported a similar result in the enzyme retention of 66% and 69.2% on SiO₂ nanoparticles at the same optimum condition of 30°C, pH of 5. However, the agitation speed and incubation time on both studies differ, where at 115 rpm and 66 h incubation time resulted in 117% efficiency. While at 35 rpm and 30 h incubation time resulted in 144% efficiency. This indicates that the higher the speed and incubation time, the enzyme efficiency will decrease. They also tested the enzyme's stability and reported 92-97% retained activity after 17 cycles of usage. The main advantage of this method is the reusability, due to the stability and insoluble properties of SiO₂ as an immobilizer. Moreover, this method was found to be cost-effective and highly suitable for commercial uses (Homaei *et al.*, 2013).

FURTHER DEVELOPMENTS

While there have been new developments in the production of fungal xylanase, such as fungal purification using Aqueous Two-Phase Affinity Partitioning (Fakhari *et al.*, 2017) and polyelectrolyte precipitation (Podestá *et al.*, 2019), there is still room for further development. For example, there is potential to investigate other polymers to improve the polyelectrolyte precipitation that was reported by Podestá *et al.* (2019). The astounding variety of fungal species that can be used to produce

xylanase as indicated by the many varieties of fungal species described in various parts in this review also indicates the potential for new undiscovered fungal species to be researched for their xylanase producing capability. This also includes potential research on upstream parameter optimization for these potential new fungal species. There is also potential value in researching fungal xylanase solid-state fermentation in various pilot-scale bioreactors as this review has only discovered the research using fixed-bed bioreactor by Castro *et al.* (2015). One of the last suggestions is to further study the effect of genetic engineering to further increase the fungal xylanase production (which was not covered in this review) such as the research that has been performed by Cayetano-Cruz *et al.* (2016) and (Liu *et al.*, 2019).

CONCLUSION

This review has explained fungal xylanase and some of its applications, as well as its upstream and downstream production process and the factors that affect those production processes. There are many factors that affect the upstream production of fungal xylanase, such as the identification of fungal species or strains that are capable of producing a high yield of xylanase. Factors affecting upstream production mainly include the parameters of the fermentation process. While this review may have given an idea of how several factors may affect fungal xylanase's upstream production process, preliminary studies are still recommended to those who aim to set up new fungal xylanase production operations. The same also applies to the downstream processing part of the fungal xylanase production. Future effort should be directed towards optimizing the downstream

processing of fungal xylanase and developing new methods that might be more efficient. Preliminary studies are recommended as the factors that need optimization depends on the need or specification of the user. Finally, future research may consider thoroughly reviewing how genetic engineering may further increase fungal xylanase production aside in addition to optimizing the upstream and downstream parameters.

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