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CRITICAL PHYSICAL PARAMETERS FOR OPTIMUM RECOMBINANT PROTEIN PRODUCTION IN YEAST SYSTEMS

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MINI REVIEW

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Abstract

Yeasts become the most preferred expression system for the production of recombinant proteins which play an important role in the development of biopharmaceutical products, antibodies for disease treatment, and enzymes for the food industries. The ability to grow in simple media, and ease of genetic manipulation with the benefits of typical eukaryotic expression which include protein processing, folding, and post-translational modifications, have pushed them as one of the emerging hosts for recombinant protein production. Furthermore, yeasts are additionally quicker, easy to use, and cost-effective with high yield production in comparison to higher expression hosts. The effective productivity of the recombinant proteins is also influenced by the external parameters. This paper reviews different optimization methods of the recombinant protein production for several factors such as pH, temperature, media, agitation rate, inducer, inoculum size and induction time using one factor at a time (OFAT), Response Surface Methodology (RSM) and Artificial Neural Network (ANN). This review highlights the current studies regarding the optimization of the recombinant proteins expressed in three different yeasts namely; *Saccharomyces cerevisiae, Komagataella phaffii,* and *Yarrowia lipolytica*. These are the critical parameters which can be used to optimize the recombinant protein in yeast systems. The purification methods used to purify the proteins are also discussed for each system.

INTRODUCTION

For the past decades, yeasts have been used as an expression host for the production of various kinds of recombinant proteins. Out of many other existing eukaryotic hosts, yeasts require lower nutritional demands in comparison to other higher host systems such as insects, plants, and mammalian cell lines [1], [2]. Previously, the bacteria *Escherichia coli* is considered the most preferred host system for recombinant protein production because of the availability of genetic tools, simple growing techniques with rapid growth. But, there are several bottlenecks present when dealing with *E. coli* expression system like the protein are mostly misfolded and accumulated as inclusion bodies [3], requires selection markers in the culture medium [4], unable to reach high cell densities due to acetate by-product [5] and does not confer a post-translational for eukaryotic proteins [6]. In order to combat those limitations in *E. coli*, yeast combines simplicity, fast-growing and ease of genetic manipulation with the capacity to undergo desired eukaryotic post-

translational modifications for biologically recombinant proteins [3]. Selection of yeast integrated plasmid has generated antibiotic-free culture for recombinant protein production with great stability [7]. The yeast *Saccharomyces cerevisiae* is often used for the proteins which require significant post-translational modification rather than using bacterial hosts that need to pass through an additional in vitro process for the introduction of post-translational modification. It is also the first eukaryotic system to have its genome sequenced [8]. It has been used for the production of various pharmaceutical proteins since the early days of genetic engineering techniques [9]. *S. cerevisiae* can produce soluble recombinant proteins and provides an alternative approach for many problematic proteins which are not appropriately folded that lead to precipitation and form inclusion bodies within *E. coli* [6]. However, performance of high-mannose type N-glycosylation of the modified protein limits the use of yeast which confers a short half-life that can lead to reduce efficacy for therapeutic use [2]. Apart from that, when dealing with integrated plasmid, a low copy number of plasmid can be observed in yeast genome. Thus, required huge numbers of screening for the colonies which can produce the highest expression level [10].

Lately, many new species of yeasts have been discovered and studied for their use in industrial applications. Among the existing yeast species, *Yarrowia lipolytica* and *Komagataella phaffii* (previously known as *Pichia pastoris*) are widely used as alternative expression hosts to produce recombinant proteins. *Y. lipolytica* has been considered to be a good candidate host for the production of homologous and heterologous proteins because of the availability of whole-genome sequence and genetic tools, and the ability to utilize low-cost hydrophobic substrates with good secretory capability. The proteins produced from this host are used in multiple biotechnological applications for example, an enzyme lipase is one of the most important metabolites produced by *Y. lipolytica* due to its wide range of technological applications in pharmaceutical and food industry, or in the production of oils that are enriched with essential fatty acids, or to produce and store lipids for biofuel productions [11], [12]. Pang et al. (2019) reported the production of limonene from the genetically engineered *Y. lipolytica* strains using waste cooking oil as a carbon source increased up to 20-folds titre compared to glucose. It has shown its functions and significance as an expression system for its utilization in metabolic engineering and different biotechnological applications [13].

The attempts of using *S. cerevisiae* and *Y. lipolytica* have been reported to have several limitations regarding the problems of hyperglycosylation, low transformation efficiency and high occurrence of negative transformants [14]. Subsequently, *K. phaffi* becomes the most common and effective system in molecular biology for the production of both the secreted and intracellular recombinant proteins [15] and suitable for large scale production of heterologous proteins [16]. To enhance the performance of a system and to minimize the experimental effort, optimization of experimental culture conditions is needed. Several statistical techniques were successfully applied in different studies as an optimization procedure [17]. Among the popular optimization methods, One-factor-at-a-time (OFAT), Response Surface Methodology (RSM) and Artificial Neural Network (ANN) are the most widely used techniques in different systems.

CONVENTIONAL VERSUS NON-CONVENTIONAL YEAST

Earlier, *S. cerevisiae* which is considered as conventional yeast has been utilized as a platform for the production of various recombinant proteins and has been a commercial success. Moreover, being generally regarded as a safe (GRAS) organism which lacks significant endotoxin unlike bacteria, it becomes a favoured expression system for biopharmaceutical productions [18]. However, some of its properties need to be improved for significant production such as for increasing the capacity of protein secretion for commercial use [19]. For example, *S. cerevisiae* performs complex process to convert into a mature protein which includes different steps of post-translational modifications (PTM) such as protein folding, additional glycosylation, sorting, and secretion [8]. As reviewed earlier, to obtain maximum protein yields with high quality, engineering of secretion pathways to understand the cellular reaction would be significant to increase the secretion capacity. However, the approach is only applicable for a single or a few proteins and it is difficult to establish an improved platform for heterologous protein expression [8].

The limitations of using conventional yeast as an expression system could be overcome by alternating the host system. In recent years, various recombinant proteins are being produced using non-conventional yeasts such as *Y. lipolytica* and *K. phaffi* [20]. However, engineering of non-conventional yeasts is difficult due to lack of advanced genome editing tools, and incomplete study of their genetics and metabolic pathways. Yet they provide many potential advantages over conventional yeast in terms of desired product profile [21]. As reviewed earlier, *Y. lipolytica* has been shown to have more secretory capacity per cell (40 times) than *S. cerevisiae* [21]. *K. phaffi* is advantageous over other hosts, for example, the recombinant *Komagataella* can grow in a wide pH range from 3.0 to 7.0, although the highest yield of hEGF (human Epidermal Growth Factor) is obtained in the pH range of 6–7. This feature is an extra advantage of the host where a wide range of pH can be used for the optimization of the recombinant protein production [22]. Different kinds of proteins expressed by *S. cerevisiae*, *K. phaffi*, and *Y. lipolytica* and the optimum parameters for expression were summarized in Table 1.

METHODS FOR OPTIMIZING THE RECOMBINANT PROTEIN PRODUCTION

The optimization of the recombinant protein production is commonly performed either at the levels of gene expression (expression level) or fermentation (process level) or with the selected factors from both levels. The importance of integrating computational approaches and experimental methods for recombinant protein production in optimization is highlighted.

Experimental Approaches

Classically, One-factor-at-a-time optimization (OFAT) is a frequently used optimization method in recombinant protein expression to investigate the effect of one independent variable at a time while other factors are held at constant levels [23]–[25]. This method requires numerous sets of experiments to complete the analysis and involved time-consuming and challenging. However, OFAT has been applied in situations where a general understanding of the influence of certain factors on recombinant protein expression. Hence, statistical optimization has been used to relate with the interactions among the studied factor.

The design of experiments (DOE) is an optimization technique, especially, response surface methodology (RSM) is used to obtain the optimal response by statistical analysis. RSM optimizes the effecting factors statistically and determines the optimum values with different factors with less experimental requirements [26]. This approach is carried out in two steps. Firstly, the screening experiment in a minimal set of trials was conducted, which is commonly based on full or fractional factorial designs (FFD), Placket-Burman design (PBD) [19], or Taguchi's robust design. Next, the designs of RSM-based, for example, Central Composite Design (CCD) or Box-Behnken Design (BBD) have been applied for the optimal recombinant protein expression. CCD is implemented with RSM to estimate a second-degree polynomial model for analyzing the effects of individual variables on the response (dependent variables) [27], [28]. The analysis of variance (ANOVA) is important for validating the quadratic model and examining the significance and acceptability of the model [29].

Multiple studies have been conducted using RSM. Alidee et al. (2017) had illustrated the optimization of Selenium (Se) enriched *S. cerevisiae* for maximum biomass and Se synthesis by RSM. The experiment was conducted using three parameters, including initial sodium selenite (Na_2SeO_3), initial pH and incubation temperature, which resulted in a maximum biomass of 6.69 g/L and 3766.07 ppm of Se yield [30]. The other study demonstrated the optimization through CCD with the aid of Minitab16 as an optimal design for recombinant phytase expression in *K. phaffi* using parameters; pH, methanol concentration, and temperature, and determined the effect of their interactions on phytase activity [26]. A recent study showed the optimization of culture conditions to improve the production of α-galactosidase in *S. cerevisiae* (ScAGal) by the recombinant strain BJ3505/ YEpMEL1His using the experimental factors; glucose concentration, aeration, pH and culture time; and the α-galactosidase activity as the response. It was found that 80% aeration, OD_{600} of 10 (as the overexpression of protein did not harm the cells), and 1.5% (w/v) glucose at pH 6 showed the maximum response (66U/mL) when grown for 190 h [28].

Gandhi et al. [22] reported by using OFAT approach, the expression of recombinant α amylase SR74 was successful in *K. phaffi,* with a two-fold increase in enzyme productivity over that of *E. coli,* after optimizing the external parameters such as pH and temperature [31]. *S. cerevisiae* has also shown enhanced protein production in a defined medium when supplemented with amino acids but the medium needed to be optimized for high-density cultivation [32]. However, optimization by OFAT is costly, labour intensive and often impractical when a large number of variables needs to be examined [33], [34]. Moreover, the interactions of the factors would affect the metabolism inside the cells [25]. As reviewed earlier, it could not reveal the information regarding the interactions among the experimental parameters limiting the use of this method [35]. Besides, changing the inputs may lead to unpredictable responses on the outcomes of other factors that are unreliable [36].

Computational Approaches

Another sophisticated technique is using computational tools where they are developed based on the specific properties of gene- or protein-based factors that influence recombinant protein production. These bioinformatics approaches are applied to reduce the cost and avoid laborious experimental procedures. The design experiment ranging about 5 to 60 in numbers collectively corresponds to the protein-based factor. Examples of the computing methods include (i) dynamic programming, (ii) statistical methods involving linear regressions, and (iii) algorithms such as Artificial Neural Networks (ANN). However, the strength and applicability of these computational tools depend on the selected critical factors and the computing methods [35].

ANNs are well performed in pattern recognition and random relationships of mass variables modelling [37]. The interconnecting neurons carry a value known as a weight that takes part in learning the networks [38] i.e. adjusting and transmitting data from one node to the other nodes [39]. The algorithm is a widely used supervised ANN learning model which represents a series of input and output data to the system. The successful application of ANN has been reported in several studies; such as in

Table 1. Examples of recombinant proteins expressed in *S cerevisiae*, *K. phaffi* and *Y lipolytica*

Yeast	Recombinant Protein	Source	Substrate	Factors				No. of fold increased	Function	References
				pH	Temp(°C)	Time(h)	Mol.Wt (kDa)			
S. cerevisiae	Human serum albumin	*S. cerevisiae* (2805)	YPDG medium + 2% galactose + 0.1 Potassium phosphate	6.3	30	72	67	2	Plasma expander in surgery	[60]
	Hirudin	Plasmid YEG-HIR525α	Yeast extract (16g/l)	5.5	-	ND	6.9	ND	Anticoagulant	[74]
	Human transferrin	DYB7(pDB3237)	BMMD (buffered minimal medium dextrose	6.2	-	-	75	12	Erythropoiesis	[72]
	Human antithrombin III	*S. cerevisiae* BY4741	Minimal media + 20% galactose and 10% raffinose	7.5	22	84	58	ND	Regulate blood coagulation	[19]
	Hepatitis B Small Antigen (HBsAg)	Yeast strain BY4741	YPAD medium	ND	37	ND	ND	2.12	Vaccine for hepatitis B	[75]
	α-galactosidase	α-galactosidase	p‑nitrophenyl‑α‑d‑galactopyranoside	6.0	22±2	216	55	20	Degradation of RFOs	[28]
K. phaffi	Human interferon α2b	pPICZαA(Invitrogen) cloned in *E. coli* (DH5α)	BMGY + BMMY + 0.5% methanol	6.0	20	ND	18.8	ND	Antiproliferative and immunomodulatory effects	[47]
	Ice Binding Protein (IBP)	*Leucosporidium sp*. (LeIBP)	YPD + 50% glycerol (fed-batch)	6.0	25	144	25	37	Binds to ice crystal and inhibit its gowth	[43]
	α-glucosidase	*Aspergillus Niger*	BMMY medium + 0.5% methanol	5.5	50	ND	57	1.57	Produce isomalto oligosaccharides	[76]
	Pectin methylesterase inhibitor (PMEI)	Kiwi fruit	BMMY medium + 0.5% methanol + 0.8% sorbitol + 0.05% oleic acid	-	30	-	20	ND	Food additives for control of endogenous plant PME activity	[77]
	Phytase (PPHY)	*Pichia anomala*	3% methanol + 1% YE + 0.3% tween-80	4.0	60	ND	420	21.8	Improve dietary phosphorus availability in monogastrics	[27]
	T1 Lipase	*Geobacillus zalihae*	YPTM + 2% methanol	9.0	-	48	45	ND	Catalyse the hydrolysis of long chain triglycerides	[63]
	β-glucosidase	*Fungus Periconia sp*.	BMMY/BMGY	6.0	30	168	-	4	Hydrolyse oligosaccharides	[78]
	Human epidermal	pPIC9K-hEGF cloned in *E. coli* (DH5α)	BMMY medium + 0.5% methanol	7.0	29	60	6.2	10	Promote the generation of new	[22]

	growth factor (hEGF)							epithelial & endothelial cells	
	Invertase	*S. cerevisiae* SUC2 gene	BMGY + BMMY	4.8	60	96	60	3.83	Hydrolysis of sucrose	[79]
	Human C-reactive protein (CRP)	pPICZaA/CRP	BMY + 1% glucose	7.4	28	48	23	ND	Used in diagnosis of various diseases	[71]
	Lipase	*Thermomyces Lanuginosus*	BMGY + methanol 3%	8.0	30	72	35	ND	Food industry	[80]
	Human bispecific diabody (BsDb)	pPICZαA(Invitrogen) cloned in *E. coli* (DH5α)	BMGY + BMMY + 1% methanol	4.0	37	96	51	ND	Potential agent for cancer therapy	[45]
Y. lipolytica	Human interferon α2b	*Y. lipolytica* (JMY1852p)	GNY medium + glucose (20g/l) + oleic acid (1.25g/h)	5.0	28	19	19.4	19	Antiproliferative and immunomodulatory effects	[61]
	Invertase	*S. cerevisiae* invertase SUC2 gene	YNB medium + 0.5% glucose + 0.1% YE	5.0	37	72	60	ND	Hydrolysis of sucrose	[81]
	Pro transglutaminase	*Streptomyces hygroscopicus*	YPD medium + 50% glycerol (w/v)	5-6	40-45	1	48-120	8.8	Catalyse crosslinking between γ-carboxyamide groups	[82]
	Thermophilic β-mannanase	*E. coli* (DH5α)	YM medium + 60% glucose (w/v)	5.0	70	-	48	2.2	hydrolyse the 1,4-b-D-mannosidic linkages	[46]
	Lipase	*E. coli* (DH5α)	YPM broth	6.0	40	ND	35	ND	Hydrolysis of lipids	[11]
	D-limonene	Waste cooking oil	Waste cooking oil (20%)	5.72	20	216	60	20	nutraceutical and pharmaceutical industries	[13]
	L-limonene	Waste cooking oil	Waste cooking oil (20%)	-	-	-	-	-	-	-

*ND = Not Determined

biomarker identification and classification [39]; gene expression and DNA binding information [40]. ANN also has been applied in optimizing the production of enzymes such as protease, laccase, polygalacturonase, arginine deaminase, and hydantoinase [37]. The expression of recombinant proteins for the production of recombinant human epidermal growth factor (hEGF) has been shown significant effects after optimizing the combination effects of the pH and the temperature using ANN in recombinant *K. phaffi* [22].

SIGNIFICANT PARAMETERS INVOLVED IN OPTIMIZATION

Optimization of external factors is crucial for the efficient production of recombinant protein since every protein is different in terms of their properties and growth condition. Thus, it is important to select the best parameters to achieve maximum yield and high activity level of the desired recombinant protein. Variations in the composition of the culture medium, pH, temperature, concentration of inducer, as well as the point and length of induction may also influence the productiveness of yeast cultures [26]. Moreover, typically the same enzyme might show different characteristics if the expression host is altered. Thus, optimization needs to be done in order to find their optimum condition where the enzyme works best [41].

Temperature

Temperature is an important external factor for the growth and metabolic activities of microbial cells [13] since it correlates with all biological enzyme catalysis processes [42]. It is also one of the crucial operating parameters in fermentation processes in yeasts and plays an essential position for the optimization of the recombinant protein production as it does not only affect the yield produced but also the integration of the protein structure. Changes in temperature also influence the activity of the enzyme (by increasing or decreasing the protein concentration) and the folding of protein structure. Optimization of this parameter is a prerequisite for the recombinant protein to increase their stability where it works best [26], and it affects the synthesis of extracellular enzymes by altering the physical properties of the cell membrane. Increasing the temperature from the optimum would decrease in the protein yield since the cell could not be stable at higher temperatures, releasing proteases from dead cells and create protein misfolding [22]. However, in some cases, production of protein does not affect drastically by the induction temperature for example, the production of recombinant ice-binding protein from *Leucosporidium sp.* (rLeIBP) in *K. phaffi* has an optimal temperature at 25°C and no detection of severe proteolytic degradation in various temperatures (15, 20, 25 and 30°C) since the enzyme activity was stable in all the temperatures [43]. Several studies have been reported for the expression of recombinant proteins which are greatly influenced by the effects of temperature such as amylase [44], β-galactosidases [17], human epidermal growth factor (hEGF) [22], human bispecific diabody (BsDb) [45], β-mannanases [46], and lipase [11]. One study proposed that decreasing the temperature (30-20°C) during methanol feed dramatically increased the recombinant protein (INF-α2b) yield by reducing the proteases levels secreted in the medium using *K. phaffi* as the host system [47].

Media (Minimal Versus Complex)

Culture media are key environmental factors in cell growth and significantly affect various metabolic reactions. Particularly, phenotypes of microbial cells are regulated by the concentration of ions contained inside the medium, and variations in the amounts of elements may influence the changes in phenotypic characters of microbial strains [48]. Minimal and complex media have been used for research and industrial purposes in which complex media contain undefined components with high contents of carbon and nitrogen sources as compared to minimal media [49].

In the previous study, differences between the metabolic status of *S. cerevisiae* and *E. coli* grown in the minimal and complex media had been demonstrated to investigate the effect of both the medium whereby the fatty acids and sugars were abundant in minimal media while the complex medium had more amino acids for the protein synthesis and rapid growth. This could be used as a piece of information for improving the yield of amino acids or sugar and fatty acids as a precursor or final product in industrial microbial fermentation [49]. Both the media can be used for research and industrial purposes, higher amount of carbon and nitrogen sources in complex media results in higher growth rate and different level of final outcomes in microbial fermentation [49]. For biopharmaceuticals, minimal or defined growth medium would be beneficial for downstream purification and regulatory documentation. However, the organisms grow slowly than on nutrient-rich complex medium since cell synthesizes all the metabolic intermediates in minimal media [50]. Therefore, it is necessary to design and optimize an efficient medium particularly for the products intended for pharmaceutical applications [51]. A new rich defined medium (RDM) have been designed for *K. phaffi* to increase the complexity that would help in cell growth and the transcriptomics study to understand the underlying metabolic processes, and demonstrated the expression of three heterologous proteins (namely; hGH, human growth hormone; IFN α-2b, and G-CSF, granulocyte colony-stimulating factor) of higher titres than those expressed in standard complex media [50]. In another study, to maximize the production of hINF α-2b and to minimize the activity of endogenous protease, a defined culture medium was designed specifically for *Y. lipolytica* [52]. An efficient medium, GYN, was proposed and optimized using statistical experimental design for large

scale heterologous protein (hIFN α-2b) production by *Y. lipolytica* which allowed the medium without any non-defined composition requirement where the increased in biomass (416-fold) as well as the biological activity (2-fold) were observed [51].

Inoculum Size

Size and age of inoculum used also influence fermentations affecting growth rate, yield, cell morphology [53], the final product quality [54] and effect on the microbial economics [29]. Therefore, it is important to optimize the inoculum size. A number of studies have shown that 2% inoculum size as optimal condition for the expression of proteins [29], [53], [55], [56]. For example, inoculum of more or less than 2% have shown reduced in fructofuranosidase (FFase) production in *S. cerevisiae* [53], as the concentration of yeast inoculum might influence competition for space and nutrients in the medium [57]. One study reported the optimization of inoculum size using different ranges from 0.5-5.0% for the production of rhIFN-γ (human interferon gamma) in *K. phaffi* and found that 2% inoculum significantly maximized the production (2.1 mg/L) which suggested that increased in inoculum size did not improve in the final product [55]. Carolina et al. (2014) reported the enhance production of ethanol as the inoculum amount was raised from 0.4 to 4 g/L using the industrial strains *S. cerevisiae* UFPEDA 1238 (95%) and UFPEDA 1324 (76%) [49].

Inducer

The productivity of recombinant protein in yeast is also dependant on the presence of inducer as one of the effective factors which promotes the expression of genes [26]. Variation in the concentration of inducer may enhance or decrease the productiveness of the yeast cultures. For example, a study was reported the effect of galactose concentration on GAL regulated cloned gene expression in recombinant *S. cerevisiae* whereby, a galactose concentration of 0.4% was sufficient for high level expression of β-galactosidase and furthermore, increasing the inducer concentration to 2.0% substantially improved β-galactosidase synthesis [58]. For the expression of heterologous proteins, different strains of yeasts rely on the inducible promoters not only the constitutive promoters. Since the utilization of strong constitutive promoters lowers the secretion efficiency due to the accumulation of misfolded proteins namely, insulin precursor, and α-amylase [59], several researches showed the advantageous of using inducible promoters which allowed to control the gene expression levels by optimizing the concentration of inducible molecules as reviewed earlier [9], [21]. Previous articles reported that galactose was widely used both as a carbon source and inducer for the growth of *S. cerevisiae* [2]. The endogenous GAL1 and GAL10 promoters are mostly used as inducible promoters with the inducer molecule, galactose for the production of recombinant proteins in *S. cerevisiae* [9]. However, the periodic feeding of galactose (2% (w/v)) into the culture medium for the maximal induction of GAL10 had enhanced the degradation of secreted recombinant human serum albumin (HSA) into the medium. It was observed to decrease the extracellular pH below 4.5 when a significant amount of HSA start to be susceptible to degradation [60]. It was suggested from the observation that the periodic feeding of galactose caused acidification of the culture medium that might result in the extracellular degradation of HSA. This finding had shown the negative effect of using higher percentage of inducer for induction.

In a study, evaluation of three different sources (olive oil, methyl oleate, and oleic acid) were demonstrated as inducers and oleic acid was found to significantly induced the production of hIFN α-2b using *Y. lipolytica* as an expression system [61]. Being oleaginous yeast, several promoters have been developed based on the oleic acid-inducible promoters such as POX1 and LIP2. However, significant inducible promoters are still lacking for the expression of gene in *Y. lipolytica* because utilization of these promoters is difficult especially in large-scale bioreactor due to the hydrophobic nature of the inducers such as fatty acids or triglycerides. Identification and characterization of a novel inducible promoter for EYK gene (erythrulose kinase coding gene) were reported from *Y. lipolytica* which could utilize erythritol and erythrulose as free inducers increasing the expression of EYK1 gene which showed low expression levels when induced by glucose or glycerol [62].

In *K. phaffi,* the heterologous gene is commonly cloned under the control of an AOX (alcohol oxidase) promoter that can be induced in the presence of methanol [21]. Formaldehyde dehydrogenase (FLD) and alcohol oxidase (AOX) promoters were found in *K. phaffi* [63] and most of the methylotrophic yeasts possess similar inducible promoter in their methanol utilization pathways [21]. Among all the promoters, AOX1 is the most preferred for the recombinant protein expression as it is one of the strongest and most regulated promoters since it can integrate expression plasmids in its own genome in one or more specific sites, and able to culture strains in high-density fermenters [64]. As reviewed earlier, the utilization of a mixture of carbon sources during the methanol induction phase alleviated the metabolic burden derived from heterologous protein production and increased recombinant protein productivity [21]. Optimization of methanol concentration was done for the production of recombinant lipase and found 1.5% (v/v) to be the best concentration for induction which required higher concentration as compared to other lipases that only required 0.5% (v/v) for the optimum induction from a number of previous studies [59]. Utilization of methanol, being a highly combustible and hazardous substance, might be undesirable for large-scale fermentation. In the previous study, it was reported that a novel methanol-free *K. phaffi*

strain was engineered with the modification of transcription factors of AOX1 promoter. And demonstrated the high expression of Insulin Precursor (IP) (2.46g/L) induced by glycerol with a decrease in oxygen consumption and heat evolution compared to the wild-type in methanol [65].

Agitation Rate

Better control of rotation speed also favours cell growth and higher yield production because it influences the mass transfer of gases (O_2 and CO_2) for the continuous supply, in and out of the growth medium [13], [29]. It also maintains homogenous chemicals and physical conditions in the medium [42]. The speed of agitation has direct effects on the shape and biomass dispersal in the culture medium [37]. It is important to optimize the agitation speed since high rotation may damage fragile microorganisms and will affect product formation while low speed may influence in viscosity leading the mass transfer reduction [42]. As reviewed by Subramaniam et al. [32], increasing biomass and product concentrations enhanced foam formation which could interfere with normal conditions, therefore measures for foam reduction should be considered for high-density cultivation. In the previous study, Pang et al. [13] investigated the effect of agitation on the accumulation of D-limonene and L-limonene by the engineered *Y. lipolytica* strains (Po1g KdHR and Po1g KlHR) and established 250 rpm as the optimum agitation speed and also suggested that high speed in shake culture flask was important for sufficient oxygen supply to maximize the limonene production in *Y. lipolytica.* Recently, one research was performed on the optimization of agitation speed on the fermentation of *A. pullulans* for pullulan yield. Increased in agitation (up to 200 rpm) reduced the size while significantly increase in the overall colony counts and showing visible effects of agitation rate on size, shape, and distribution in the medium [37].

pH

Extracellular pH is a crucial parameter to obtain maximum microbial activity in the culture media. The changes in pH directly influence membrane potential, proton gradient, and consequently substrate utilization and product profile. Therefore, it is important to optimize the pH closer to the microbial physiological pH which would be significant for gene expression, enzyme performance, signalling mechanisms, and the overall biological activities of the organism [13]. Optimization of pH by Yaping et al. [46] revealed that genetically engineered thermophilic β-mannanases i.e. ManA and ManB in *Y. lipolytica* showed highest activities at the pH of 5 and 7 respectively. Furthermore, activities of ManA were stable over a pH range from 4 to 9 and ManB from 6 to 9 after incubation at 37°C for 1 h [46]. In another study, the highest activity of recombinant lipase produced in *Y. lipolytica* was shown at pH 6 and it maintained more than 86% of its residual activity at pH 4-8 when incubated for 30 min [11]. Pang et al. [13] found the highest titers of limonene at pH 5.74-7.98 from the engineered *Y. lipolytica* strains which showed to be the optimum pH (5.74) for the production of limonene. In addition, the pH of culture media would also affect the activities of various proteases that may degrade the heterologous proteins [22].

Incubation Time

Incubation time for a fermentation process is vital for the growth of microorganism and production of recombinant proteins utilizing the medium content [37] for increasing expression level overtime during incubation, while the products may be degraded under some circumstances if the induction duration is overdue [15]. Therefore, it is also important to maintain the optimum incubation time which the enzyme can be in its maximum activity. For example, recently in 2019, Efigenia et al. showed the expression of α-galactosidase in *S. cerevisiae* (ScAGal) achieved its maximum activity (21.09 U/mL) when it was incubated for 144 h at pH 6. Whereas the enzyme activity was drastically reduced up to 3.45 U/mL when incubated for 48 h under the same culture conditions [29]. This showed that the incubation time was the crucial positive effect for the expression of ScAGal compared to glucose concentration and aeration. Most of the microbial expression system would express at the stationary phase as compared to exponential phase. Because, during exponential phase like yeast expression systems in general, the cell culture will focus on generating the mass and start to be induced once the carbon-nitrogen sources are depleted. Time and feeding strategy (with the respective carbon sources) are the key factors in controlling the recombinant protein expression.

PURIFICATION OF THE RECOMBINANT PROTEIN IN DIFFERENT SYSTEMS

Development of rapid, cost-effective methodologies for the purification of recombinant proteins in the absence of contaminants is of great value. Recombinant proteins expressed in yeasts can be readily purified, unlike bacterial systems which require additional steps to lyse the harvested cells followed by the removal of cell debris and purified the biomolecule from the clarified cell homogenate by precipitation and/or chromatography [66]. The recombinant proteins in yeast normally will be secreted into the culture medium using the α-factor secretion signal [67]. The purification steps in the downstream processing represents 70% of the total costs and the methodologies based on chromatography is the most prominent [68] that can purify proteins from a less expensive residue with high-resolution capacity [17], [66]. The affinity chromatography, due to its high selectivity, is commonly used in the purification of

heterologous proteins in small and large scale production [68].

For column chromatography, one major disadvantage of using yeast lysates is the viscosity of cell lysates with polysaccharides found in the cell wall and membrane debris requiring an additional filtering step before passing through the column. To decrease the viscosity, Xie et al. [69] described the effective purification system by diluting the cell lysates with purification buffer (1:5) which require minimal expertise and provided high protein solubility [69]. Another study compared the expression and purification of human glutamic acid decarboxylase (GAD67/65 hybrid) from *S. cerevisiae* and *K. phaffi* using metal affinity chromatography [70]. Particularly, the histidine tag (H6) has been commonly used for simplifying the purification of recombinant proteins from bioprocess mixtures [68], [71]. The hybrid was incorporated with the hexa-His tag in order to purify large amounts of GAD from the two host systems, however, the addition of H6-tag or the choice of host strain did not affect the catalytic properties of the enzyme [70]. Yet another group described the difficulties to obtain pure protein without the purification tag and therefore considered recombinant *S. cerevisiae* strain with his tag (BJ3505/YEpMEL1His) as the preferred system for the production of α-galactosidase (ScAGal) [28]. If the expression of the protein is intended for therapeutic purpose, then the purification must be extremely refined. Mallu et al. [19] successfully expressed and purified recombinant human antithrombin (rhAT) from *S. cerevisiae* with size exclusion chromatography (SEC) followed by ion-exchange chromatography (IEC) which showed similar purity with that of heparin affinity columns.

Among the yeast expression systems, *K. phaffi* is noted for easy purification techniques with the advantages of high yield production, genetically stable expression strains, the potential for recombinant protein to secrete free into the culture medium, and inexpensive culture conditions [42], [65]. For example, recombinant human C-reactive protein was expressed in *K. phaffi* strain (X-33) and purified with 97% purity (single species yield according to SDS-PAGE analysis) using a single step HisTrap HP-affinity column chromatography procedure [71], unlike that of *S. cerevisiae* which required two steps chromatography procedure [72]. Similarly, recombinant xylanase ($Xyn11A_{AOX1}$) from *K. phaffi* was purified by one step of anion exchange chromatography with a specific activity of the enzyme and a higher yield than the expected amount for the mature $Xyn11A_{AOX1}$ [73]. However, due to the presence of secreted proteases in the medium, which may lyse or degrade other proteins, most of the high cell-density cultures are having problems. Hence, some studies have shown a reduction in protease levels by maintaining low temperature, enhanced the yield of active protein in *K. phaffi* [47].

CONCLUSION

The choice of a suitable host system to produce recombinant protein is subjected on the structure of the target protein and requirement of the post-translational modifications. Yeast expression system has received acceptance as a vital host organism for the production of recombinant protein. Critical parameters and optimization strategies are clearly discussed in this review for three commercial yeast expression systems; *S. cerevisiae, K. phaffi* and *Y. lipolytica*. In current recombinant protein expression using microbial system, yeast becomes a more efficient and economic platform which can support the increasing demand.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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